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IMMUNOLOGIC, GENETIC, AND HEMATOLOGIC STUDIES OF H-2 INCOMPATIBLE PARABIOSED MICE

by

© DANIEL WHITE DRELL

University of Alberta

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled IMMUNOLOGIC, GENETIC, AND HEMATOLOGIC STUDIES OF H-2 INCOMPATIBLE PARABIOSED MICE submitted by DANIEL WHITE DRELL in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Immunology

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ABSTRACT

Parabiosis of adult DBA/2J (H-2d) mice with adult (DBA/2J x C3H/HeJ)F1 (H-2d/H-2k) mice results in long term survival of 44% of such pairs, the complete takeover of the F1 lymphoid (spleen and bone marrow) and erythropoietic (circulating RBC) systems by parent type cells, and specific unresponsiveness in situ to C3H/HeJ skin. The survival to 100 days, the severity of the polycythemia-anemia syndrome associated with classical parabiosis intoxication, and the extent of the parental takeover reaction are all influenced by genes within the H-2 complex of the F1 "target" partner. Genes in the K to I-E interval play the most important role. The takeover reaction requires parental immunocompetence to the target haplotype.

Immunological responsiveness remains in the parabionts, as judged by ^{51}Cr release assays, GVH reactivity, and the killing in vivo of a ^{131}I-iododeoxyuridine labeled tumor histocompatible with C3H/HeJ. Parabiont spleen cells show full reactivity upon stimulation with C3H/HeJ alloantigens and do not inhibit the stimulation of normal DBA/2J cells. Intact parabionts also show tumor destruction in situ even when not deliberately restimulated with C3H/HeJ cells. Parabiont serum gives rapid tumor destruction on passive transfer, and complement dependent antibody titers of 1:10 to 1:20 are present against lymphoid target cell antigens coded for by genes in the H-2K to H-2I-E interval. These

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titers are sensitive to 2-mercaptoethanol. Despite this antibody, parabiont F1 skin grafts show no delay in rejection by normal DBA/2J recipients.

The retention of immunological reactivity, the importance of immunogenetic disparity, and the presence of anti-"graft" antibody suggest a possible role for immunological enhancement in parabiosed mice.

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ABBREVIATIONS

MLR:	Mixed Lymphocyte Reaction
ALS:	Anti-lymphocyte Serum
GVH:	Graft-Versus-Host
GPI:	Glucose Phosphate Isomerase
RBC:	Red Blood Cells
2-ME:	2-Mercaptoethanol
^{131}I-RI:	^{131}I-iododeoxyuridine labeled RI (H-2k) tumor cells
i.p.:	intraperitoneal
i.v.:	intravenous
ARA:	Anti-receptor Antibodies
ADCC:	Antibody-dependent cellular cytotoxicity
PEC:	Peritoneal Exudate Cells
PBS:	Phosphate Buffered Saline
FCS:	Fetal Calf Serum
MEM:	Minimal Essential Medium
PHS:	phytohemagglutinin
EBSS:	Earle's Balances Salt Solution

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INTRODUCTION

I. Tolerance and Mechanisms

In 1953, Billingham, Brent and Medawar reported their now classic experiments on the induction of specific transplantation tolerance in adult mice, by the neonatal injection of histoincompatible lymphoid cells (Billingham et al., 1953). These experiments showed that specific nonreactivity to foreign tissue grafts, in this case to skin, could be induced by an experimental manipulation which did not otherwise affect the immunocompetence of the manipulated animal. Responsiveness to skin allografts not genetically identical to the tolerance - conferring lymphoid cells was normal and for the permanent acceptance of the skin graft which was genetically identical, no nonspecific immunosuppressive treatments were needed.

These experiments have spurred on a search for other ways in which specific unresponsiveness to tissue grafts can be established in adult animals. Particularly, ways have been sought to induce nonreactivity in adult animals by pretreatments which do not require knowing far in advance the genetic identity of the donor; such a restriction would severely limit the application of new methods to human patients in need of organ transplants. The ideal is a pretreatment regimen, applied shortly before organ grafting and requiring simple and accessible donor materials, which

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would induce specific unresponsiveness without in any way reducing the immune responses of the graft recipient to other foreign antigenic substances.

The work which will be presented in this thesis describes and begins to characterize one such model for the induction of unresponsiveness to tissue antigens in adult, otherwise immunocompetant, mice.

As knowledge of the immune system has increased, it has become apparent that the observed absence of a response to transplantation antigens can be mediated by a number of not necessarily mutually exclusive mechanisms. These mechanisms may be conveniently divided into four types: deletion, active suppression, lack of stimulation, and enhancement. This categorization is not meant to be exhaustive, but general enough to encompass the most commonly discussed ideas, these also being the ones on which the most work has been done.

The first concept is that of the specific deletion of the agents of immune reactivity. Originating in the work of Owen (1945) with vascularly anastomosed cattle twins, developed into a general concept by Burnet and Fenner (1949), and dramatically confirmed by the experiments of Billingham, et al., (1953), the <u>in utero</u> or neonatal exposure of a mouse to cells bearing foreign alloantigens can lead to specific and long lasting tolerance to those alloantigens. Frequently, these animals do not respond <u>in</u>

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vivo or in vitro, to stimulation by cells from the strain to which tolerance was induced, although responses to all other alloantigens appear normal (Wilson, et al., 1967; Schwartz, 1968; Wilson and Nowell, 1970; Brent et al., 1972; Beverly, et al., 1973; Brooks, 1975; Silvers, et al., 1975).

It is possible also to create tolerant states in adult animals, states which appear to be described by the complete absence of responsive cells. Characteristically, lethal irradiation must be given to the recipient, which is then restored with bone marrow or fetal liver cells from a genetically non-identical donor (Sprent, et al., 1975; von Boehmer, et al., 1975; Tulunay, et al., 1975; Grant, et al., 1972). Such animals do not give rise to cytotoxic cells against the strain to which tolerance was induced and mixed lymphocyte reactions (MLR) are greatly reduced (Sprent, et al., 1975) or absent (von Boehmer, et al., 1975). Thus by physically destroying the host lymphoid system and replacing it with the stem cells for a new one, tolerance can be induced which seems to result from the elimination of specifically reactive cells.

Both in neonatal tolerance models and adult tolerance states, immunologic reactivity can be concealed by the presence of active suppression. In neonatally tolerized rats, Roser and Dorsch (1979, and Dorsch and Roser, 1975) have found specific thymus-derived suppressor cells, as have Hasek and Chutna (1979). Rieger and Hilgert (1977) and

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Hilgert (1979) have evidence that injection of allogeneic spleen extracts into neonatal mice can induce suppressor cells specific for the tolerizing alloantigens. Droege (1975) reported a suppressor cell implicated in transplantation tolerance in chickens. Still others have reported that non-cellular "blocking factors", of undetermined composition, could specifically interfere with immune reactivity in vitro (Bernstein, et al., 1975, Wright, et al., 1975, Hellstrom and Hellstrom, 1974). These blocking factors may be complexes of antigen and antibody (Sjogren, et al., 1971).

Reports of suppressor cells in adult tolerant states to virtually every antigen have recently proliferated throughout the immunological literature (see Transpl. Rev. vol. 26, 1975, also Singhal and Sinclair, 1975; Pierce and Kapp, 1976). Alloantigen specific suppressor cells can arise following total lymphoid irradiation (Strober, et al., 1979) and have been found in other models as well (Jirsch, et al., 1974). Suppressor cells have been claimed to arise during allogeneic pregnancy (Clark and MacDermott, 1978, and Suzuki and Tomasi, 1979) although there is disagreement about their relevance (Wegmann, et al., 1979). Kilshaw, et. al., (1975b) suggested that there may be a suppressor cell in their somewhat complicated tolerance model, which involves injections of liver extract and B. pertusis vaccine, then skin grafting, then injections of anti-lymphocyte serum (ALS), all in adult mice. Although suppressor cells may be

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involved in some forms of unresponsiveness both in neonates and adults, the cautionary words of Steinmuller (1978) must be acknowledged, that many of these studies require irradiated or ALS treated animals in which to demonstrate the existence of suppressors and thus give rise to unavoidable questions of interpretation and significance. The hallmark of active suppression, by cells or serum, is its transfer to a fresh recipient which is then unable to express a reactivity that it normally can express, i.e. the phenotype of unresponsiveness is dominant over responsiveness in the recipients of the transferred materials.

A third way in which a response to a graft might be prevented is if the graft itself is lacking in some immunostimulatory ability required to trigger the response against it. Much evidence that cells of lymphoid and hemopoietic lineage are especially stimulatory to responsive lymphoid cells has been accumulated (Steinmuller, 1967; Billingham, 1971; Lafferty and Woolnough, 1977). This special significance of cells of lymphoid and hemopoietic origin will be referred to again (Section VII.D) because the absence of such "passenger cells" may account, in part, for some of the observations in this thesis.

A final major way in which immune rejection can be avoided, frequently indefinitely, is by antibody-mediated enhancement (reviews: Voisin, 1971b; Feldman, 1972;

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Carpenter, et al., 1976). Several reports (Voisin, et al., 1972; Hasek, et al., 1975; Gutmann, et al., 1978) conclude that enhancing antibodies can act to maintain neonatally induced tolerant states, but most enhancement studies pertain to adult animals grafted with allogeneic tumors or organs (see above cited reviews).

Tumor grafts have been studied ever since Flexner and Jobling (1907a, 1907b) reported that prior injection of a rat with heated tumor emulsion 10 days before inoculation of the test sarcoma resulted in promotion of the growth of the tumor. Subsequent work by Casey (1932, 1933) confirmed the enhancement (as he termed it) of tumor growth by extracts inoculated prior to the administration of live tumor, and demonstrated tumor type specificity (Casey, 1933); that is, material from a mouse carcinoma would enhance the growth of a subsequent graft of the same carcinoma, but would not enhance the growth of a mouse sarcoma graft. Kaliss and Snell (1951) demonstrated that extracts of normal tissues would enhance and that enhancement was not a property just of neoplastic tissues. Kaliss and Molomut (1952) demonstrated passive transfer of enhancement with serum. Snell (1954) linked tumor enhancement with the major histocompatibility complex in the mouse, H-2 (see Klein, 1975 for a review of the mouse H-2 system), showing that antigens determined by the H-2 complex were important components of the enhancement phenomenon.

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In the survey which follows, enhancement will be considered in detail. Enhancement can occur in adult animals following appropriate pretreatments, is mediated by antibody, permits the retention of anti-graft cell mediated responsiveness, varies in degree with the specific genetic incompatibilities between graft and recipient, and, in some models, need not even be deliberately induced (Voisin, 1971b; Feldman, 1972; Carpenter, et al., 1976). As will be described in Results, the parabiosis model which is the subject of this thesis, also involves the presence of antibody, is associated with the persistence of anti-"graft" cell mediated responsiveness and some of the sequelae of parabiosis vary with the immunogenetic incompatibilities involved. In this parabiosis model, the deletion of reactive cells clearly does not account for the observed results, nor, apparently, does a suppressor cell detectable in the assays employed. The absence of stimulatory passenger cells may play a role, as may the antibody and for this last reason especially, the literature of immunologic enhancement appears the most relevant. Much of this literature pertains to tumor enhancement, but recently, more has appeared on organ graft enhancement and this work will be emphasized.

II. Definitions and Terminology

It will be convenient for the discussions to follow, if certain terms are defined now. Since much of the literature on immunological enhancement pertains to studies on tumor systems (see Kaliss, 1969; Snell, 1970; Voisin, 1971; and

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Feldman, 1972 for reviews) the definitions frequently cited reflect this.

The broadest definition of immunological enhancement is Voisin's (1971): "the mechanism whereby an antibody (or an immune reaction) promotes the persistence of the corresponding antigen and the integrity of the cytological structures which support it, by preventing it from inducing or undergoing (or both) immune rejection." The antigen may be either tumor tissue or normal tissue including an entire organ. Since many reviews (see Snell, 1970 and Feldman, 1972) particularly emphasize the importance of antibody as the mediator of enhancement, that emphasis will be maintained here as well. Voisin's definition does not restrict a priori how the enhancing antibody may act. From start to finish, an immune response is a complicated process and the potential to affect it exists at virtually any point in the sequence. Enhancement as understood here will encompass any interference by specific antibody in the induction, recognition, processing, or effector stages of an immune response.

Active enhancement refers to any procedure or combination of procedures in which antigenic material, such as cells, or cell extracts, or a prior graft of tissue or tumor, is administered to an animal prior to inoculation of that animal with the test graft (Feldman, 1972). Several examples of possible active enhancement have already been

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cited (Flexner and Jobling, 1907a; Casey, 1932; Kaliss and Snell, 1951).

Passive enhancement refers to experiments in which the recipient of a graft is injected with serum from an animal whose immune system has already confronted tissue genetically identical with the graft. An important implication here is that the serum donor need not have rejected the grafted tissue; he need only have been exposed to it. An important test of the enhanced status of a host is the successful passive transfer of serum from the enhanced host to a new one. Because the amounts of serum required for the induction of enhancement are often uncertain, as is the concentration of "active ingredient", (see below, section VI) this test is frequently not described. An example of passive enhancement is the study of Kaliss and Molomut, (1952).

A number of reviews (Levey, 1971; Voisin, 1971a; Elkins, et al., 1974; Brent, et al., 1976) have considered the relationship of immunological enhancement to transplantation tolerance. Here, a "tolerant" animal is one that fails to express the form of immune reactivity, whether antibody mediated or cell mediated, which is being assayed. No implication of a mechanism is intended, since, as briefly reviewed before, several different ones may be invoked. A tolerant animal may not be totally incapable of a specific immune reaction, only unable to express the one under test.

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Thus the failure of graft rejection which can follow enhancement induction is regarded as "tolerance" only when graft rejection is the assay; if the expression of an antibody response were the assay, such animals would frequently appear non tolerant. Thus tolerance and enhancement are perhaps best regarded as a set and one of its subsets respectively; enhanced animals may appear tolerant by some assays, but tolerance can result from mechanisms other than enhancement. Tolerance, as operationally defined here, is a phenotype while enhancement is a mechanism.

III. Early Attempts at Enhancement of Normal Tissue Grafts

Billingham and Sparrow (1955) first reported some success with deliberate attempts at prolonging normal tissue allografts in adult animals. In these experiments, prolongation of skin grafts in rabbits was achieved by pretreatments with donor epidermal cells. This success at active enhancement was extended by Billingham, et al., (1956). Using inbred mice which offered the advantages of unvarying genetic backgrounds and the consequent reproducibility, they achieved slight prolongation of skin grafts across H-2 differences by injections of lyophilized donor strain kidney or spleen tissue. Although the earlier rabbit experiments (Billingham and Sparrow, 1955) were thus confirmed, this work with inbred mice indicated that skin was a difficult tissue to enhance using an approach then known to work well with tumor grafts in inbred mice.

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Guttmann and Aust (1961) achieved very considerable skin graft prolongation using i.v. injection of large numbers ($3-3.4 \times 10^8$) of donor spleen cells, again with inbred mice. Prolongation was specific and could be achieved with smaller numbers of injected cells in immunogenetically less disparate strain combinations. This work was confirmed by Brent and Gowland (1962) who also showed that i.v. injections were an absolute requirement (i.p. injections sensitized) and that unresponsiveness was to a degree progressive with greater antigen dose. Both Guttmann and Aust (1961) and Brent and Gowland (1962) preferred the interpretation that their pretreatments had led to the induction of true tolerance in which potentially responsive host lymphoid cells had been eliminated.

Brent and Medawar (1962) showed that passive transfer of immune serum, in a manner analogous to that used with tumor grafts (Kaliss and Molomut, 1952), could prolong donor skin graft survival. This prolongation was slight but reproducible. Using non-inbred guinea pigs, Nelson (1962) also passively enhanced skin grafts, with serum from guinea pigs which had themselves rejected donor skin grafts. Nelson (1962) also showed donor specificity of this effect and its removal upon absorption of the serum with donor lymph node cells. In contrast to the earlier studies with mice, Nelson did not interpret his results to follow from induction of true tolerance. Clearly graft prolongation resulted from the presence of an activity rather than from the absence of

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reactivity.

Halasz and Orloff (1963) tried active enhancement on an entire organ graft, pretreating mongrel dogs with donor whole blood prior to kidney transplantation. A marked prolongation of graft survival was seen, indicating that an entire organ might benefit from attempts at immunomodification by enhancement prior to grafting. Thus these experiments gave encouraging indications that grafts of normal tissue could be actively or passively enhanced, and that the phenomenon was not unique to tumors.

IV. Experimental Enhancement

The emphasis of this review will be on the experimental enhancement in adult recipients, of grafts of skin, heart, kidneys and occasionally, endocrine tissue. These tissues offer technical advantages both in performing the grafts and assaying their subsequent fate. Successful enhancement of these types of grafts has been achieved by active pretreatment of the recipient with materials of donor strain derivation, by passive transfer of anti-donor antisera, by the combination of active and passive procedures, and, in a few instances, spontaneously in the absence of any pretreatments.

1. Active Enhancement

Cells, cell extracts, a prior graft of tissue, or any combination of these, in which the recipient is exposed to antigenic material from the future donor strain prior to

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grafting, constitute active immunization leading to enhancement when such pretreatment results in graft prolongation (Feldman, 1972).

a) Skin

Several examples of active enhancement of skin grafts have already been noted (Billingham and Sparrow, 1955; Billingham, Brent and Medawar, 1956; Guttmann and Aust, 1961; Brent and Gowland, 1962; and Nelson, 1962). Other investigations have demonstrated that active immunization with nonviable donor material such as frozen and thawed lymphoid cells (Mayer et al., 1965), alcohol and heat killed spleen cells (Heslop, 1966) or lyophilized spleen cells (Chutna, 1968) can give prolongation of skin allografts, and, where it was checked, this prolongation was donor specific (Heslop, 1966). In the studies of Heslop (1966) and Chutna (1968) subsequent passive transfer of serum from actively enhanced animals further enhanced donor strain skin grafts.

Brent's group has thoroughly studied the induction of specific unresponsiveness in CBA mice given A strain liver extract i.v., Bordetella pertussis vaccine i.v., then skin grafting, then three injections of anti-lymphocyte serum (ALS) (Brent et al, 1973; Kilshaw et al, 1974; Pinto et al., 1974; Brooks et al., 1975; Kilshaw et al., 1975a; and Kilshaw et al., 1975b). The unresponsiveness which this extended procedure induces is strain specific, the mice do

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not show detectable hemagglutinating or cytotoxic anti-donor antibodies, and the unresponsive state can be broken by the adoptive transfer of 10^8 syngeneic (CBA) lymph node cells (Kilshaw et al., 1974). Other strain combinations can also be made to work, the grafted mice have precursors for cytotoxic cells to the donor, recipient cells can give graft versus host reactions in F1 hybrids presenting the "tolerated" alloantigens (Brooks et al., 1975), and these unresponsive mice also have cells which react in mixed lymphocyte cultures with donor strain cells. No blocking factors could be shown in their serum (Kilshaw et al., 1974a). In one report, evidence was presented suggesting that a T-suppressor cell was present (Kilshaw et al., 1975b).

The use of a nonspecific agent such as ALS may also play a key role in the induction of enhancement. A number of studies have shown that unresponsiveness to skin or organ grafts can follow specific active or passive immunization (vide infra) combined with ALS (Monaco, 1970, and Koene, et al., 1975, for skin grafts; Goodnight, et al., 1976, for heart grafts; and Batchelor, et al., 1972, and Lie, et al., 1977, for kidney grafts).

What most of these reports have in common is that the immunization procedures should lead to the production in the recipient of anti-donor antibodies. Indeed, Shapiro et al. (1961) found that (AxC3H)F1 skin grafts were "permanently"

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accepted by C3H recipients actively conditioned by alternating i.v. and i.p. injections of F1 spleen cells whereas just i.p. injections of F1 spleen cells resulted in virtually none of the injected C3H mice accepting F1 skin grafts, confirmed by Brent and Gowland (1962). I.v. injections would tend to shift the developing immune response towards antibody and away from cell-mediated immunity (Voisin, 1971a).

b) Hearts

Active enhancement of heart allografts can follow pretreatment of the recipient with donor whole blood (Jenkins and Woodruff, 1972; Marquet and van Bekkum, 1973) or even, in mice, by an interstrain pregnancy (Heron, 1972) which, it is known, can give rise to anti-paternal strain antibodies (Kaliss and Dagg, 1964; Goodlin and Herzenberg, 1964). The work with active enhancement of heart grafts parallels much of the work with active enhancement of skin grafts, except that the heart graft experiments generally are more successful (Jenkins and Woodruff, 1972, 1973; Marquet and van Bekkum, 1973).

In these experiments, recipient anti-donor cell mediated immunity persisted, often for considerable lengths of time after grafting (Jenkins and Woodruff, 1973; Heron, 1972; Marquet and van Bekkum, 1973). Anti-donor antibody was present and, upon passive transfer, graft recipient serum could passively enhance a donor strain heart graft (Heron,

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1972). The work of Jenkins and Woodruff (1972, 1973), suggested that in their model immunogenic components present on lymphocytes but not on RBC were critically important for the induction of enhancement. In addition, enhanced heart grafts regrafted to secondary recipients were rejected. This suggested that the establishment of the enhanced state was due largely to alterations in the responsiveness of the recipient, rather than to "graft adaptation", that is, changes in the "appearance" the graft presents to the host immune system (Section VIII.1.C).

c) Kidneys

THe work with active enhancement of kidney grafts, usually in the rat, has been the most successful. A number of groups have actively enhanced rat kidneys (Ockner, et al., 1970a, b; Guttman, et al., 1972; Fabre and Morris, 1972a, b, 1975; Zimmerman, 1977; Desi and Ruszkiewicz, 1977). In all of these models except Zimmerman's (1977), parental strain rats (usually Lewis, Rt1.A1) were pretreated with whole blood i.v. from an Rt1.A incompatible donor strain (BN, Rt1.An or DA, Rt1.Aa) 7 days prior to grafting with a kidney from an F1 hybrid donor ((Lewis x BN)F1 or (Lewis x DA)F1). Zimmerman (1977) grafted fully allogeneic BN kidneys into Lewis hosts pretreated with BN whole blood.

Generally, these experiments indicated that kidney graft enhancement was immunologically specific and did not extend to skin grafts (Ockner, et al., 1970a, b; Fabre and

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Morris, 1975; Desi and Ruszkiewicz, 1977; and see Section V.4). Indicators of cell mediated reactivity such as the GVHR remained (Ockner, et al., 1970b, Fabre and Morris, 1972b). In Ockner's, et al., (1970b) experiments, serum from actively immunized rats enhanced on passive transfer, but serum from actively immunized and grafted rats was not tested; in the experiments of Fabre and Morris (1972b), passive transfer was unsuccessful. Fabre and Morris (1972b) also found that upon retransplantation, enhanced kidneys were eventually rejected but with a delay in the onset of this process, indicating that some graft adaptation may have occurred. When fresh F1 kidneys were grafted back into the enhanced donors, they were uniformly accepted, emphasizing that the enhanced state was intrinsic to the host. Desi and Ruszkiewicz (1977) found cytotoxic anti-donor IgM antibodies 20 days after grafting but these had declined to undetectable levels beyond 4 months after grafting.

d) Active Enhancement: An Overview

Surveying active enhancement of grafts of skin, heart, and kidney, a general set of characteristics emerges. A number of factors such as timing, dose, route of administration, and the nature of the immunizing material are important. Also important is the tissue being enhanced. As will be discussed below in section V the immunogenetic relationship of graft to host is also important. Generally 10^7 to 10^8 white blood cells, or a preparation containing about this number of lymphoid cells, injected i.v. at least

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7 days prior to grafting, will induce prolongation of a tissue graft histocompatible with the immunizing cells. This procedure is least effective for skin grafts, but considerably more effective for heart and kidney grafts. Generally, F1 hybrid grafts are easier to enhance than fully allogeneic grafts (Feldman, 1972). The phenomenon of enhancement is immunologically specific.

Actively enhanced animals demonstrate a generally consistent picture of persistent cell mediated responsiveness towards donor alloantigens, measured both by donor skin graft rejection and in various transfer tests such as the GVHR reaction and the MLC. The enhanced state is an alteration in the host but some degree of graft alteration can occur (Jenkins and Woodruff, 1973; Fabre and Morris, 1972b).

In some models, passive transfer of enhancement was achieved with serum (Heslop (1966) and Chutna (1968) for skin, Heron (1972) with hearts, Ockner et al., (1970b) for kidneys); in others (Kilshaw, et al., (1974); Jenkins and Woodruff, (1972); Fabre and Morris, (1972b)) it was not. The reasons for this inconsistency in different models are not clear but may include variation between animals, variations in the quantity of enhancing antibody in different sera, or variations in the amount of serum passively transferred.

The presence of anti-donor antibodies was not always checked. In the studies of Desi and Ruszkiewicz (1977) anti-

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donor antibodies were detectable out to 4 months after kidney grafting; they then waned to undetectable levels. As Voisin (1971a) cautions, however, the apparent presence or absence of antibodies really reflects their detection or non-detection and will be a function of the technology for assaying them.

2. Passive Enhancement

The administration of anti-donor or anti-graft antibodies to a recipient prior to grafting constitutes passive enhancement and has been extensively investigated (Feldman, 1972) since the original description by Kaliss and Molomut (1952) for tumors.The advantage it offers over active enhancement is that precise amounts of serum can be injected and thus greater reproducibility can be obtained.

a) Skin

The first attempt at pasive enhancement was Moller's (1964), who studied passive enhancement of skin grafts in inbred congenic mice. These mice differ at a limited number of defined (for the most part) genetic markers (Klein, 1975) and can be used to investigate the influence of just those markers by which the graft is known to differ from the host. Moller achieved better enhancement in a combination in which the graft differed from the host only at the H-2 region, than in a combination in which the graft differed from the host at H-2 as well as numerous non-H-2 loci. This work pointed at a possible role for other histocompatibility

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antigens besides H-2 and also suggested a possible relation between the number of incompatibilities between graft and host, and the difficulty of passive enhancement.

Jeekel, et al., (1972) enhanced B10.D2 (H-2d) skin grafts, by only a few days at best, on B6AF1 (H-2b x H-2a) recipients with 3 i.p. injections of an anti-B10.D2 (cytotoxic titer 1:512) serum at and after grafting. This prolongation was specific; B10.BR (H-2K) skin grafts were not enhanced by the anti-B10.D2 serum. Skin grafts from other H-2d mouse strains (DBA/2, Balb/c) were enhanced, indicating that the critical antigens were those of the H-2 complex. A single injection of antiserum given at the time of grafting was almost as effective as the 3 injections, indicating that the graft prolongation resulted from an early interaction of the antibody with the immune system of the host. In other studies (Sugarbaker and Chang, 1979), multiple injections were reported to be superior to a single injection; this could be due to any one of several differences in the sera used. (Section VI).

In the rat, Tilney and Bell (1974) passively enhanced (AS x AUG)F1 (Rt1.A1 x Rt1.Ac) skin on AS recipients by injecting an AS anti-AUG serum (cytotoxic titer 1:512) at and after grafting. The best prolongation of skin graft survival was a few days, comparable to results in the mouse.

One feature of attempts, both active and passive, in both rats and mice, to enhance skin grafts is their lack of

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success when complete Rt1.A or H-2 incompatibilities are involved. The oft-used CBA-A strain combination does not incorporate a full H-2 haplotype difference, only the weaker differences of the I-C to H-2D subregions (Klein, 1975). Even so, graft prolongation between these strains is a matter of a few days only (Billingham, et al., 1956; Brent and Medawar, 1962; Chutna, 1968). In other strain combinations, a prolongation by enhancement of 1-3 days is a remarkable achievement.

b) Hearts

Corry, et al., (1973a, b) using mice, and Tilney and Bell (1974) using rats, both found that passive enhancement of heart grafts was considerably easier than skin grafts. Prolongations of mouse heart grafts of 6 or more days were the norm (Corry, et al., 1973a, b) and frequently, survival beyond 100 days in the rat heart graft model (Tilney and Bell, 1974) was observed. In agreement with the work of Jeekel, et al., (1972) on passive enhancement of skin grafts in the same strain combination (B10.D2 into B6AF1 hosts), Corry, et al., (1973b) found that a single injection of enhancing serum (cytotoxic titer 1:256) on the day of grafting enhanced as well as several additional injections after grafting. The work of Davis (1979) on passive enhancement of H-2D incompatible heart grafts is in contrast to this.

In Tilney and Bell's (1974) work, secondary passive

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transfer of serum from enhanced heart grafted rats successfully enhanced both donor type heart and kidney grafts, indicating that the target antigens for enhancement were not organ specific. Cells from enhanced grafted rats gave normal local GVH reactions in appropriate F1 test animals, indicating the retention of immunologic reactivity despite enhancement.

c) Kidneys

Stuart, et al., (1968a) reported passive enhancement of (Lewis x BN)F1 rat kidneys in a small number of Lewis recipients; shortly thereafter, French and Batchelor (1969) reported similar success enhancing (AS x AUG)F1 kidney grafts in AS recipients. Stuart, et al., (1968a) used an anti-BN serum with a hemagglutinating titer of at least 1:2048 while French and Batchelor (1969) used an anti-AUG serum with a cytotoxic titer of 1:256. Hemagglutination titer and cytotoxic titer may not reflect the same activity, and neither may correlate completely with enhancement (Moore and Pareira, 1965; Voisin, 1971b); this is one of the several possible sources of variation between reports dealing with passive enhancement.

After the success of Stuart, et al., (1968a) and French and Batchelor (1969), other groups confirmed and extended their work. Lucas, et al., (1970) enhanced (Lewis x BN)F1 kidneys grafted to Lewis hosts and could even occasionally enhance fully allogeneic BN kidneys. MLR and normal

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lymphocyte transfer tests were normal using cells from enhanced rats. Cytotoxic and hemagglutinating anti-donor antibodies were detectable for several weeks after grafting; those serum preparations which enhanced especially well contained high levels of binding antibodies measured by radioimmunoassay. Thus enhancement protected the graft from immune destruction without inactivating either the humoral or cellular responsiveness of the host.

Using generally similar protocols, Stuart et al., (1971), and Strom et al, (1975) in the (Lewis x BN)F1 into Lewis strain combination, French et al., (1971) and Fabre and Batchelor (1975), in the (ASxAUG)F1 into AS strain combination, Fabre and Morris (1972) in the (DA x Lewis)F1 into Lewis combination, and Mullen et al., (1973a, b) in the (Lewis x Buffalo)F1 into Lewis combination, all passively enhanced F1 rat kidneys in parental strain hosts. In all cases, antibody was given i.v. at the time of grafting. In all cases, various assays for immune responsiveness to the F1 alloantigens remained, including the microcytotoxicity test (Stuart, et al., 1971; Enomoto and Lucas, 1972; Mullen, et al., 1973a) and the GVH reaction (French, et al., 1971; Mullen, et al., 1973a, b). Fabre and Morris (1972) found a rough correlation of cytotoxic titer in their enhancing sera and the degree of subsequent enhancement but others did not find this (Lucas et al., 1970). Strom et al., (1975) claimed that blocking factors were present in the sera of their enhanced rats but in other systems, these could not be

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demonstrated (Kilshaw et al., (1975a), Marquet and van Bekkum (1973)). Batchelor et al., (1977) looked for, but were unable to find, a suppressor cell in passively enhanced kidney grafted rats. Finally, Tilney and Bell (1974) were able to re-enhance grafts both of heart and kidney with serum from grafted, enhanced donors.

d) Endocrine Tissue

In a number of studies, passive enhancement of endocrine tissue has been achieved. Moller (1964) passively enhanced murine ovarian grafts in ovariectomized hosts across both H-2 and non-H-2 antigenic differences. More recent work has concentrated on passive enhancement of pancreatic islet cells in streptozotocin-induced (Rerup, 1970) diabetic rats, with the eventual hope of developing a cure for human diabetes. Finch and Morris (1976) passively enhanced F1 rat islets in a fraction of hosts of either of the two parental strains used to make the F1. Fully allogeneic islet grafts were not successfully enhanced. Nash, et al., (1977, 1978) also were able to enhance F1 rat islets in a proportion of parental strain recipients.

Thus, while a reliable enhancing protocol, for pancreatic islet cells at least, has yet to be defined, endocrine tissues can be passively enhanced.

e) Passive Enhancement: An Overview

Most of the features of passive enhancement are identical with those of active enhancement. Anti-donor

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serum, of hemagglutination titer greater than 1:2000 (Stuart, et al., 1968a) or of cytotoxic titer of 1:256 or greater (French and Batchelor, 1972; Tilney and Bell, 1974), in appropriate amounts, usually 1-2 mls, administered i.v. at grafting, will enhance. F1 kidney grafts especially, but also F1 heart grafts, will be protected. Fully allogeneic grafts are much more difficult.

Passively enhanced animals demonstrate a consistent picture of persistent cell mediated responsiveness towards donor alloantigens, measured by MLR, normal lymphocyte transfer tests (Lucas et al., 1970), the microcytotoxicity test (Stuart et al., (1971), Enomoto and Lucas (1972), and Mullen et al., (1973a)), and the GvHR assay (Mullen et al., (1973a, b), French et al., (1971)). Certainly the similarities between the active and passive enhancement states are impressive and suggest that the mechanisms in the host probably overlap to a considerable degree.

The exact significance of the microcytotoxicity test is controversial. Blair and Lane (1975) suggest that it may more accurately measure an antibody dependent function, perhaps antibody-dependent cellular cytotoxicity (ADCC; Perlmann and Holm, 1969). If so, this would be valuable information to acquire from animals bearing enhanced grafts and might usefully supplement passive transfer as a test for the enhanced state. Since, for enhancement studies, there is ample evidence from other assays for the retention of cell-

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mediated reactivity, the use of the microcytotoxicity technique for this purpose may not be necessary.

3. Combined Active and Passive Enhancement

By combining active and passive enhancement procedures, several groups have prolonged grafted hearts and kidneys in rats. Marquet, et al., (1976, 1977) prepared and injected antigen: antibody complexes before grafting (WAG x BN)F1 hearts into BN recipients. The premixed complexes gave superior enhancement to either antigen or antibody, given alone. This prolongation was WAG specific, did not extend to (WAG x BN)F1 skin, and co-existed with normal anti-WAG GVH reactivity. Anti-WAG antibodies were not present.

Tilney's group (Tilney, et al., 1978; Baldwin, et al., 1978) enhanced (Lewis x BN)F1 heart grafts in Lewis rats, by giving antigen and antibody a day apart, 10 days before grafting. This treatment diminished the level of cell mediated immunity as measured by ^{51}Cr release and no ADCC, hemagglutinating or complement-dependent antibodies were detectable. The combined treatment was most effective when the injected cells contained both white and red blood cells (Baldwin, et al., 1978).

The most extensive and detailed work on antigen: antibody complex enhancement of organ grafts has been done by Stuart and colleagues in the (Lewis x BN)F1 into Lewis model (Stuart et al., 1968a, b, 1969, Stuart, 1976, Stuart

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et al., 1976a, Weiss, et al., 1978 a, b, c). Initially, these studies demonstrated permanent survival of grafted F1 kidneys into Lewis rats given 10^8 F1 spleen cells i.v. the day before grafting, and an injection of anti-BN serum (hemagglutination titer 1:2048) at the time of grafting (Stuart et al., 1968 a, b). The enhancement by antigen and antibody was specific, and, although no data were given, low levels of circulating antibody were noted (Stuart et al., 1968b). When retransplanted to new Lewis hosts, enhanced F1 kidneys were rejected with a slight delay in the onset of this rejection; when fresh F1 kidneys were grafted into enhanced and previously kidney grafted Lewis rats, these secondary F1 kidney grafts were routinely accepted (Stuart et al., 1969). These results parallel exactly those of Fabre and Morris (1972b) and Jenkins and Woodruff (1973) using actively enhanced animals.

More recently, Stuart et al., (1976a) and Weiss et al., (1978a, b) have altered their protocol for enhancement, to incorporate the 10 day delay also used by Tilney, et al., (1978). Kidney grafts are routinely accepted and maintain completely normal function. Cells from graft recipients do not give direct cytotoxicity in the ^{51}Cr release assay and do not cause lethal GVH disease in sublethally irradiated (Lewis x BN)F1 rats (Stuart et. al., 197). When enhanced grafted rats were used as recipients for a local host-versus-graft test, near normal reactivity to both specific (Lewis x BN)F1 and third party (Lewis x ACI)F1 cells was

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seen. MLC reactivity against BN cells was normal with enhanced rat spleen cells and, after stimulation in MLC, enhanced rat spleen cells developed cytotoxic lymphocytes to BN strain target cells. Thus anti-donor effector cell precursors persisted after enhancement but were not triggered (Weiss et al., 1978, a, b, c). No blocking factors for any of the cell mediated assays were seen in the serum of enhanced rats. The only alteration in the immune system of the enhanced grafted Lewis host was the apparent absence of memory cells for the anti-donor cell mediated response (Weiss, 1978c).

These authors interpret these results to be due to anti-receptor antibodies (ARA), described by Ramseier and Lindemann, (1975), which peak 10 days after giving antigen:antibody complexes. A more detailed discussion of ARA is presented in Section VI.5.

Concerning antigen plus antibody mediated enhancement in general, there is little that could be added to what has already been said about active and passive enhancement separately. In some systems, combined active and passive enhancement is more effective than either alone, but the resulting profile of reactivities in the enhanced grafted host very closely resembles that in the actively or passively enhanced host. It seems plausible that whatever is happening in active or passive enhancement happens also in combined enhancement.

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What all these models appear to share is the formation either in vitro (Marquet, et al., 1976, 1977) or in vivo (Tilney, et al., 1978; Stuart, et al., 1968a, 1976a) of antigen: antibody complexes. Such complexes may play a role in active or passive enhancement as well, although the kinetics of complex formation would vary. Antigen: antibody complexes are known to affect immune reactions in vitro (Schwartz, 1971; Hellstrom and Hellstrom, 1974). Certainly in all the enhancement models discussed so far, the opportunity for complexes to form has been present, even if only at the time of organ grafting in the passive enhancement models.

4. Spontaneous Enhancement

In several systems, involving kidney and endocrine tissue grafts, anomalous survivals of these grafts into untreated recipients have been reported. These may be examples of enhancement because certain parameters of the host immune response were present and these were among the same parameters persisting after deliberate enhancement.

Both Salaman (1968) and White and Hildemann (1968) reported that spontaneous rat kidney graft survival across both minor (White and Hildemann, 1968) and major (Salaman, 1968, 1971a, b) histocompatibility barriers could occur. This spontaneous survival was asymmetric, that is, AS kidneys (Rt1.A1) survived in AS2 recipients (Rt1.Af), but not vice versa (Salaman, 1971a). In marked contrast to most

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deliberate enhancement models, these kidney graft tolerant rats also accepted donor strain skin grafts (Salaman, 1971a). Cells from grafted AS2 recipients showed essentially normal GVH reactions (Bildsoe, et al., 1971), and MLR with donor and third party cells was said to be normal (Paris, et al., 1978). Passive serum transfer was unsuccessful.

In the mouse, Russell and colleagues (Corry and Russell, 1973; Skoskiewicz, et al., 1973; Russell, et al., 1975, 1978a, b) have studied kidney grafts from donors bearing limited H-2 differences. Both B10.BR (H-2k) and B10.D2 (H-2d) kidney grafts into untreated B6AF1 (H-2b x H-2a) hosts usually survive (Corry and Russell, 1973), while in other strain combinations incorporating a full H-2 difference, virtually all grafts are rejected. In the two successful donor-recipient combinations, prolonged kidney survival often leads to extended donor skin graft survival, although it is usually not permanent (Skoskiewicz, et al., 1973). MLC and GVH reactivities persist, cytotoxic anti-donor T lymphocytes develop from the MLC, and low levels of anti-donor antibody are detectable (Russell, et al., 1975, 1978a). The grafted kidney still presents accessible target antigens and can be damaged by the injection of sensitized anti-donor lymphoid cells (Russell, et al., 1978b). No serum blocking is detectable (Russell, et al., 1978a).

Many features of these kidney grafting models are similar to actively, passively, or antigen:antibody induced

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enhancement. Kidneys, in particular, can be permanently enhanced; hearts infrequently (Corry and Russell, 1971) and skin never. Cell mediated responsiveness is retained, some indications of antibody remain (Russell et al., 1975), and the kidney remains antigenic (Russell et al., 1978b). The major difference in these models is the profound decrease reported in reactivity to subsequent donor strain skin grafts; either the initial interaction of the grafted kidney in some unknown way affects the quality of the host immune response, or this decreased reactivity has been noted preferentially in these studies.

Similar spontaneous survivals have been seen for endocrine tissue grafts by Linder (1962) with mouse ovarian grafts, Cock (1962) with chicken ovarian grafts, and Russell and Gittes (1959) and Gittes, et al., (1964) with rat parathyroid and pituitary tissue grafts. In much of this work, the immunogenetic relationships between donor and recipients were not clear since inbred animals were not available and the reported data are not extensive enough to establish that enhancement was acting. However, Cock (1962) did notice the presence of anti-donor antibody in some of his grafted chickens which is suggestive of a role here for enhancement. Another possibility is that endocrine tissues are intrinsically easier to graft than other types of tisues (Billingham and Parkes, 1955; Krohn, 1955).

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V. Immunogenetic Variables in Enhancement

A number of variables, which may be grouped as immunogenetic, influence the establishment of enhancement towards histoincompatible organ or tissue grafts. Among these are the species of animal used, the degree of histoincompatibility between donor and recipient, the strains of animals involved, and the type of organ or tissue being grafted. Very little is known about why these variables exert as important an influence as they obviously do on the outcome of experimental organ transplantation.

1. Species

Most of the enhancement work cited up to this point has been from experiments with rats. Other animals used have included mice, dogs (Halasz and Orloff, 1963), chickens (Cock, 1962), rabbits (Billingham and Sparrow, 1955), guinea pigs (Nelson, 1962), and even subhuman primates (Marquet, 1979). While the rat offers certain advantages (size and consequently greater technical ease of microsurgery, and the existence of a large number of inbred strains (Gunther and Stark, 1977)), enhancement is possible in the mouse as well (Corry et al., 1973a) and an even larger assortment of inbred strains is available (Klein, 1975). Another factor often suggested is the resistance of the rat to hyperacute rejection but this cannot entirely account for the frequency of enhancement induction to organ grafts in rats. Chavez-Peon, et al., (1971) found that rat kidneys grafted into

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mice given anti-rat serum could be hyperacutely rejected; also, rabbit kidneys grafted into rats given anti-rabbit antibody were hyperacutely rejected. Thus rats could generate hyperacute rejection responses to xenografts and rat kidneys as xenografts were susceptible to this reaction. Guttmann (1974) showed that rat heart allografts into presensitized recipients could be hyperacutely rejected; however, the only report of the hyperacute rejection of rat kidney allografts followed the infusion of guinea pig complement (French, 1972). The addition of alloantibody directly to the rat kidney in situ brings about pathology which appears very similar to rejection but this reaction takes 10 days to develop and is therefore not hyperacute (Straus, et al., 1971). Thus the antibody mediated response to a kidney allograft in the rat may be atypical and this may contribute to the frequency of enhancement of this organ graft.

2. Histocompatibility

That F1 hybrid organ grafts are easier to enhance has been a consistent observation of many investigators and is now regarded as a general principle of enhancement (Thoenes, 1975, Carpenter et al., 1976). Strom et al., (1973) found that anti-BN cytotoxic cells caused about twice the release of ^{51}Cr from BN strain target cells as from (Lew x BN)F1 target cells, and that anti-BN antibody was about twice as effective at suppressing in vitro cytotoxicity of anti-BN killer cells against F1 targets relative to BN targets. This

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roughly 2-fold difference was interpreted to be due to a difference in antigen density on F1 versus fully allogeneic target cells.

The antigens considered by Strom et al., (1973) were rat major histocompatibility antigens of the Rt1.A system (Gunther and Stark, 1977). Other, non-Rt1.A antigen systems are known to influence enhancement as well. A limitation in any discussion of the role of these antigens is that the definition of rat histocompatibility systems lags far behind the definition of the mouse H systems.

In the mouse, enhancement in tumor systems is not dependent on a particular complex of histocompatibility antigens or on types of tumors. Moller (1964c) and Bubenik, et al., (1965) have reported tumor enhancement in a syngeneic system with antibody to tumor-associated antigens and Boyse, et al., (1962) were able to enhance a leukemia by using sufficiently small amounts of antibody. Leukemias and other lymphoid tumors are known to be extremely sensitive to antibody-mediated damage (Winn, 1962).

There is an asymmetry, noted in skin and heart grafting in mice, which pertains to subregions within the mouse H-2 complex. McKenzie and Snell (1973) observed that passive enhancement of skin grafts was more successful if the graft was different from the host at the K and I subregions of the H-2 complex than if the graft differed at the D end of H-2. This appeared paradoxical since grafts incorporating K and I

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subregion differences were more immunogenic than D subregion incompatible grafts. As will be discussed in the next section (VI.3) this paradox may be resolved since antibodies to I region associated antigens are thought to play a key role in enhancement. To confuse the issue, however, Davis (1979), and Davis and Ferebee (1975), studying grafts of neonatal mouse hearts into the ears of adult recipients, found that grafts incorporating D end differences could generally be more successfully passively enhanced than K and I subregion incompatible grafts or entire H-2 region incompatible grafts.

Hildemann and Mullen (1973) have carefully analyzed the role of graded degrees of H incompatibility in rat kidney graft enhancement and found, as a general rule, that "the weaker the histoincompatibility, the greater the effectiveness of specific immunoblocking antibodies". This has been confirmed by Thoenes et al., (1974) working with congenic rats. Studying spontaneous enhancement of kidney allografts in the mouse (Section IV.5) Skoskiewicz et al., (1973), and Russell et al., (1978) have confirmed this as well.

3. Strain Asymmetries

It is not an uncommon observation that between two histoincompatible strains, A and B, grafts from A to B will survive longer than grafts from B to A. For rat kidney grafting, a number of such asymmetries are known. Salaman

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(1972b) reported that AS2 kidneys grafted to AS recipients were rejected in about 8 days, but AS kidneys grafted to AS2 recipients were frequently not rejected at all. Paris et al., (1978) confirmed this. Sakai (1969) however, had not observed this asymmetry with fully allogeneic kidney grafts, but had seen it with (AS x AS2)F1 kidneys; F1 grafts to AS2 recipients did much better than F1 grafts to AS recipients. Fabre and Morris (1975) found that passive enhancement of (DA x Lewis)F1 kidneys was easier in DA than in Lewis recipients. The significance of these observations is not clear; they could reflect variations in immunogenicity or in immune responsiveness.

4. Type of Graft and Technical Factors

Without exception, across major or minor histocompatibility barriers, in rats or mice, with or without deliberate enhancement, grafts of skin are the most difficult to prolong (Feldman, 1972). The three other types of normal tissue grafts which have been tried, endocrine, heart, and kidney, are all easier to enhance or achieve prolongation for (vide supra). In one study (Tilney and Bell, 1974), passive enhancement of skin, heart, and kidney grafts were compared directly and the rank order, in increasing ease of enhancement, of skin, heart, then kidney, was determined. Other studies have confirmed this (White and Hildemann, 1968; Barker and Billingham, 1970; Warren, et al., 1973; and Gunther and Stark, 1977). No obvious reason for these differences in susceptibility to enhancement has

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emerged although tissue specific antigens, such as the Sk antigen of skin in mice (Boyse, et al., 1970), differences in immunogenic expression (Isakov et al., 1979) or Ia antigens on skin cells (Katz, et al., 1979) may play a role.

Although hardly an immunogenetic variable, technical factors can affect how the host immune system first encounters the graft of tissue or organ. One possible contributing factor to the rapid rejection of skin grafts compared to heart and kidney grafts is that hearts and kidneys, upon grafting, are immediately vascularized; skin is not and this could affect the type of response made (Snell et al., 1976). Warren et al., (1973a) found that freely grafted (not directly anastomosed) neonatal Lewis rat hearts grafted to Rt1.A compatible Fischer recipients were rejected in about the same time as neonatal Lewis skin grafts; 5 of 8 adult Lewis heart grafts, which were surgically anastomosed, survived longer than 300 days. If both an adult and a neonatal heart were grafted together, the survival of the neonatal heart graft was considerably prolonged, indicating some systemic conditioning by the adult heart graft. This conditioning effect of the adult heart graft did not extend to a Lewis skin graft (Warren et al., 1973b). Serum from an adult-heart-grafted recipient, upon passive transfer, would enhance neonatal heart graft survival by about 7 days.

In a careful study using four different methods of

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grafting of (Lewis x BN)F1 skin to Lewis recipients, Wustrack et al., (1975) found that passive serum transfer enhanced skin graft survival only when host vascular connections to the graft occurred before lymphatic connections. In all other cases, in which lymphatic connections were made first, passive enhancement was without effect. In the one circumstance in which serum did enhance, the prolongation was only 5 days so, at least for skin grafts, this technical variation was not a major influence on its susceptibility to enhancement.

Using the same rat strain combination, Merriam and Tilney (1978) found that grafting F1 skin onto an alymphatic pedicle of host skin avoided acute rejection, but 16 to 18 days after grafting, the onset of rejection had begun and proceeded until total destruction of the graft was complete. Passive transfer of serum from such grafted rats, obtained 8-12 days after grafting, would slightly prolong F1 heart graft survival.

Yet another possible influence on graft fate is replacement of the vascular endothelium of the graft by host cells, for which some evidence exists in rats and dogs (Williams and Alvarez, 1969). Such a process, if it is important, might commence much sooner in immediately vascularized grafts such as hearts and kidneys, than in a slowly vascularized graft of skin. However, Burdick, et al., (1979) suggest that such host repopulation of elements in a

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graft can occur with xenogeneic skin (rat into mouse) protecting it from antibody mediated rejection. Precisely how important a role possible host repopulation of donor type endothelium in a graft plays must await more work.

Grafting technique, clearly of some importance for normally anastomosed organ grafts such as hearts, is of only very limited importance for skin. As with the other variables noted in this section, the exact pertinence of this factor is hard to judge. As has been pointed out in most reviews of enhancement (Voisin 1971a, Feldman 1972, Carpenter et al., 1976), competing factors are at work and the fate of a graft is determined by the balance of these factors.

VI. Enhancing Antibody

Antibody is the sine qua non of enhancement both in tumor systems (Kaliss, 1969, Voisin, 1971) and organ graft systems (Carpenter et al., 1976). Here, the class and subclass, antibody fragments, specificity, and comprehensiveness of enhancing antibody will be discussed. Finally, anti-receptor antibodies will be briefly discussed. As with many of the features of enhancement, certain observations have been made repeatedly by a number of investigators, suggesting general conclusions; however, there is no uniformity. Discordant observations and, therefore, controversy remain.

1. Class and Subclass

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Of the five defined antibody classes, (Spiegelberg, 1974) most studies concur that the most effective for enhancement is IgG. Studying the enhancement of mouse sarcoma SaI in B10.D2/o (H-2d) mice, Tokuda and McEntee (1967) found that hyperimmune anti-SaI serum, composed of 7S antibodies, efficiently enhanced, in contrast to an earlier, IgM containing serum. Upon starch block electrophoresis of the hyperimmune serum into "slow" and "fast" 7S fractions, the best enhancement was seen with the "slow" fraction. Tokuda and McEntee (1967) suggested this "slow" fraction might consist of antibodies of the IgG2 subclass. Takasugi and Hildemann (1969a) reached similar conclusions studying enhancement of SaI in A.BY (H-2b) mice. IgM antibodies were cytotoxic while IgG antibodies, and especially IgG2 antibodies, enhanced. Takasugi and Klein (1971) studied the enhancement in A.SW (H-2s) mice, of CBA (H-2k) tumors, originally induced with the carcinogen methylcholanthrene. They found that IgM did not enhance tumor growth, and that IgG antibodies did. The best enhancement was achieved with unfractionated IgG; IgG2, separated by ion exchange chromatography, showed the next most efficient enhancement, followed by IgG1, which had the weakest enhancing activity.

Voisin, et al., (1972) studied serum obtained from CBA mice tolerized as neonates to A strain alloantigens by neonatal injection of (CBAxA)F1 lymphoid cells, They observed that all three of three pools of serum enhanced an A strain sarcoma in CBA mice. The "tolerant" sera lacked

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hemagglutination, hemolysis, complement-dependent cytotoxicity or passive cutaneous anaphylactic activities towards A strain alloantigens, but had what was called specific synergistic hemagglutinating activity. Thus tolerant sera, while negative in direct hemagglutinating tests on A strain red blood cells, would increase the hemagglutination of these RBC if they were "developed" with a second hemagglutinating serum, described only as a "reference CBA anti-A immune serum" (Voisin, et al., 1972). When the tolerant sera were fractionated over DEAE-cellulose columns, the synergistic hemagglutination activity in one pool of tolerant serum co-fractionated with the IgA class antibodies, and this fraction enhanced an A strain tumor in vivo as well. In another tolerant serum pool, enhancement went with the IgG fraction.

In a study of the enhancement of SaI in (C57B1/6 x DBA/2)F1 mice (B6D2F1, H-2b x H-2d), Fuller and Winn (1973) tested serum obtained early, within 7-10 days, after injection of SaI into B6D2F1 mice. This serum, not a hyperimmune serum as often used in other studies, was fractionated into IgM and IgG; both were found to be cytotoxic <u>in vitro</u> and enhancing <u>in vivo</u>.

At least three studies have been done on the class of antibodies involved in organ graft enhancement. Van Breda Vriesman, et al., (1975) observed enhancement of (Lew x BN)F1 rat kidneys grafted to Lewis hosts with both whole

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anti-BN antiserum and the IgM from this antiserum. The antiserum used was gathered 8 days after grafting of an F1 kidney to an untreated Lewis rat and thus resembled the "early" serum preparation Fuller and Winn (1973) used from mice.

Jansen et al., (1975a) studied enhancement of B10.D2 skin grafts on B6AF1 hosts given anti-B10.D2 antiserum or IgG fractions from this antiserum. They observed enhancement with both IgG2 and IgG1 but IgG2 was more effective than IgG1, (consistent with earlier work noted above: Tokuda and McEntee (1967), Takasugi and Hildemann (1969a), Takasugi and Klein (1971)). Within the IgG2 subclass, IgG2a was slightly more effective than IgG2b. Both components of IgG2 would lyse B10.D2 target cells in vitro when used with rabbit complement, suggesting that enhancement may not result from solely a qualitative feature of an antiserum but from a more complex dynamic of qualitative and quantitative factors.

Studying (Lewis x Buf)F1 kidney grafts to Lew recipients, Mullen et al., (1977) found that IgG enhanced and that the IgG2a subclass was almost as effective as the whole IgG. In the Rt1.A compatible Fischer to Lewis situation, IgM from a Lewis anti-Fischer hyperimmune serum reduced kidney graft survival while IgG from this serum improved survival, or, at the very least, did not impair enhancement in this weak combination in which kidney grafts into untreated recipients do very well.

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In some experiments, quantitative factors may be concealed; enhancement, perhaps a property of any class of antibody if of the right specificity and present in appropriate amounts, may be masked by lysis. According to this idea, advanced by Moller (1963a), the quantity of antibody, regardless of class, which can completely block the antigenic receptors on the target cells should maximally enhance if the antigenic density of the target is low enough to make complement binding and lysis unlikely. Consistent with this is the apparent predominance of IgG, rather than the more lytic-efficient IgM, in enhancement. Boyse, et al., (1962) however, had found that leukemias, known to have high antigen densities and high susceptibility to complement mediated lysis (Winn, 1962) could be enhanced by very low amounts of antibody. Furthermore, sarcomas and other low antigen density, complement resistant tumors can be destroyed in vivo if enough antibody is given (Winn, 1970). The notion that enhancing antibodies are qualitatively distinct is now favored (Voisin, 1971b), although quantitative factors are still important.

Very small amounts of IgG can detectably affect immune responses (Haughton and Nash, 1969; Ryder and Schwartz, 1969); thus the efficiency of fractionation of a serum preparation tested for immunomodification is a valid concern. Furthermore, not all sera are drawn at comparable times after immunization; in some studies, hyperimmune serum was used (Tokuda and McEntee, 1967; Takasugi and Hildemann,

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1969; Takasugi and Klein, 1971), while in other studies, "early" serum was used (Van Breda Vriesman, et al., 1975, Fuller and Winn, 1973). Thus comparisons between experiments are not straightforward. Although different reports claim varying degrees of enhancement for three different classes of antibodies (IgG, IgM, and even IgA), most of these reports find enhancement by IgG. Within these reports, the IgG2 subclass is said to enhance both tumor grafts and organ grafts more effectively than other subclasses. One report even localizes enhancement to the IgG2a component (Jansen et al., 1975a), but notes that other subclasses (IgG2b, and IgG1) can enhance as well. Thus, although it may not always be the class of antibody which mediates enhancement, most studies would agree that antibodies of the IgG fraction and perhaps of the IgG2 subclass play a predominant role in enhancement both of tumor and organ grafts.

2. Antibody Fragments

In the previous section on enhancing antibody class and subclass, it was concluded that IgG2 antibodies played an important role in several enhancement models. IgG2 antibodies fix complement by their Fc regions (Spiegelberg, 1974) and so investigations have been undertaken to determine whether the presence of the Fc portion is critical to enhancing antibody. One important caution must be noted here. Many tumor enhancement studies (Moller, 1963a, b, c; Takasugi and Hildemann, 1969a, b; Takasugi and Klein, 1971) have utilized mouse sarcomas. Sarcomas are known to be

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relatively insensitive to the action of antibody and complement *in vitro*, as compared to lymphoid tumors, such as the leukemia EL-4 (Winn, 1962; 1970). Antibody devoid of its Fc portion will not bind complement and if complement participation *in vivo* is important for enhancement, the presence or absence of the Fc region will affect the results considerably depending on whether a lytic-sensitive or lytic-resistant tumor is used.

Chard et al., (1967) generated high titered Balb/c (H-2d) anti-EL-4 (H-2b) hyperimmune serum and fractionated it to isolate the IgG2 antibodies. These were then treated with the enzyme papain to generate Fab fragments. When the tumor EL-4 was mixed with these Fab fragments and injected into Balb/c mice, enhanced tumor growth was observed. It was argued that undigested IgG2 was unlikely to account for this because whole IgG2 did not enhance but instead inhibited tumor growth *in vivo*. In a later study (Chard, 1968) anti-EL-4 sera was fractionated into IgG2 and IgG1 pools; IgG2 was found to be cytotoxic for EL-4 while IgG1 showed some enhancing ability. When the IgG1 antibodies were digested into either Fab or F(ab')2 fragments, both were found to give slight enhancement of EL-4 growth in Balb/c mice.

In a xenogeneic system, studying the enhancement of a mouse Ehrlich ascites tumor in guinea pigs, Broder and Whitehouse (1968) found enhanced growth of the mouse tumor after pretreatment with F(ab')2 fragments of guinea pig

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anti-tumor IgG2 antiserum. Intact IgG2 slightly inhibited tumor growth.

The studies noted so far suggest that antibody fragments can enhance in various systems. Very recent reports contradict this almost completely.

In a study of the enhancement of Lewis (Rt1.A1) kidney grafts into DA (Rt1.Aa) rat recipients, Winearls et al., (1979) compared whole IgG and very carefully prepared F(ab')2 fragments of this IgG from DA anti-Lewis enhancing serum. Both the whole IgG and the F(ab')2 were shown to bind equally to Lewis target cells; however, while 25-50 microliters of whole IgG would enhance 80-100% of Lewis kidney grafts to DA hosts, even a 10 fold excess (500 microliters) of F(ab')2 gave only marginal enhancement. These authors concluded that this marginal enhancement could have been due to contamination of the F(ab')2 with undigested IgG and that kidney graft enhancement was Fc dependent.

A totally concordant conclusion to that of Winearls et al., (1979) was reached by Capel, et al., (1979) studying mouse skin graft enhancement. In their hands, F(ab')2 fragments of a B6AF1 anti-B10.D2 antiserum had no enhancing activity at all. In another study, this same group (de Waal, et al., 1979) found that 22 times as much of the F(ab')2 preparation from a B6AF1 anti-B10.D2 antiserum did not enhance a B10.D2 fibrosarcoma, even when the F(ab')2 was given daily. Thus enhancement here as well was concluded to

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be Fc dependent.

One likely reason that enhancement is now viewed as requiring intact antibodies is technical. The methods available for fractionating papain digested (to generate Fab pieces) or pepsin digested (to generate F(ab')2 pieces) antibodies and cleanly separating the fragments away from the undesired contaminating whole IgG, simply are better. Certainly, the recent studies show rare uniformity in their conclusion that the Fc protion is required for enhancement. Another possible factor is that the early studies used EL-4, a lymphoid system tumor known to be very sensitive to antibody plus complement (Winn, 1962; 1970).

To what purpose is the Fc required? Two possibly important functions are known to localize to the Fc region: binding of complement, and adsorption of certain antibody species to Fc receptors on the surfaces of certain types of cells, such as monocytes and macrophages, and some lymphocytes (Winkelhake, 1978). How such effector functions of the Fc region might be involved in enhancement is not known.

3. Specificity

Most systems of tumor or organ enhancement cross major histocompatibility complex differences (Rt1) in the rat (Gunther and Stark, 1977) and H-2 in the mouse (Klein, 1975)). Although less is known about the rat Rt1 region, considerable knowledge of both the mouse H-2 region and the

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human HLA complex (Klein, 1975, Snell et al., 1976) has been accummulated and neither are simple; they are complexes of a number of loci, most of which, perhaps even all of them, appear to have immunological functions. The question of which of the loci in the major histocompatibility complex is most important for enhancement has been actively studied, especially in the mouse where various H-2 subregions have been defined. The two general regions of relevance here are the classical serologically defined loci (K and D in the mouse) and the lymphocyte defined (I region) loci. This distinction is operational and not complete; I region loci can now be serologically characterized as well (David, 1976). Nevertheless, I region loci appear to have a different spectrum of involvements in immune functions than the K and D regions (McDevitt, et al., 1976, Klein and Hauptfeld, 1976).

A key finding about the mouse I region is that cell surface antigens of restricted tissue distribution have been identified and murine red blood cells do not appear to carry these "Ia" antigens (David, 1976). A number of investigations have exploited this by absorbing enhancing antisera with target strain RBC to study whether the remaining activity in the antisera includes enhancement.

In studies on rat heart graft enhancement (Davies and Alkins, 1974) and on mouse skin graft enhancement (Staines, et al., 1974; Staines, et al., 1975a, b), RBC absorbed

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antisera have been found to enhance just as well as unabsorbed antisera indicating that activity against K and D type serologically defined antigens is not required. In an extension of their studies on carefully purified enhancing IgG2 antibodies, Jansen et al., (1975b) also found that RBC absorbed antibodies enhanced mouse skin grafts as effectively as did unabsorbed IgG2. In a rat kidney graft model, Soulillou, et al., (1976) observed that RBC absorbed, or platelet (another Ia negative cell type; David, 1976) absorbed sera enhanced with full effectiveness. In a study with I region congenic mice, McKenzie and Henning (1977) localized the I subregion critical for skin graft enhancement to the I-A subregion, a conclusion hesitantly tendered by Staines, et al., (1975a) earlier.

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In parallel with emphasizing the importance of anti-I region antibodies for graft enhancement, several reports have noted that antibodies eluted off target RBC do not appreciably enhance (Catto, et al., 1977, Staines, et al., 1975a). Jenkins and Woodruff (1971) had found that antisera raised against donor rat RBC would not passively enhance heart grafts whereas antisera to whole blood would enhance. The presence of anti-I region activity would also explain the findings, noted earlier, of McKenzie and Snell (1973) that K region differences were both more immunogenic and more easily enhanced than D end differences; all the K end different combinations they studied contained I-A incompatibilities as well. Gallico and Mason (1978)

confirmed that DA anti-Lewis RBC sera would not enhance Lewis kidneys grafted to DA rats. Those sera raised against tissues which were Ia positive or could conceivably contain Ia positive cells (see the Section VII.1.D) would enhance.

Three reports slightly discordant from the above evidence on the overwhelming importance of Ia antibodies in enhancement must be noted. Davis (1977) reported passive enhancement of neonatal heart allografts in mice across just the H-2D region which is not known at present to contain an I-like locus. The conclusion from this report was that an Ia locus was not an absolute requirement for enhancement but this conclusion must remain provisional until the loci in and around H-2D are better understood. Duc, et al., (1978) studied the enhancement of the SaI tumor (H-2a) in C57B1/Ks (H-2d) mice with antisera prepared against A strain (H-2a) spleen cells and also antisera from CBA mice rejecting A strain skin grafts. The enhancement ability of the first serum was entirely absorbed with A strain RBC and the eluate from these RBC restored enhancement. The second serum, anti-skin graft, enhanced both before and after RBC absorption, as did the RBC eluate. Whether this reflected different compositions within the two antisera, or different requirements for enhancement in a tumor system was not determined. SaI is a sarcoma and not thought to carry Ia antigens.

Baldwin et al., (1978), studying combined passive and

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active enhancement of rat skin and heart grafts, found that best results followed administration of whole spleen cells as antigen; results with just white blood cells as the antigen were only "modest". When antigen was whole spleen cells, followed by RBC absorbed antibody, the results were not as good as whole spleen followed by unabsorbed antibody. Thus this study found that both anti-serologically-defined activity and anti-I-region activity were important for enhancement.

A final type of specificity should be noted; specificity towards organ or minor H determinants. Tilney and Bell (1974) passively enhanced (AS x AUG)F1 rat hearts and kidneys in AS recipients. Serum from these enhanced recipients would, on secondary passive transfer, enhance both F1 hearts and kidneys regardless of whether the source was an enhanced kidney recipient or an enhanced heart recipient; this suggested that organ specificity was not an influence in these experiments. However Marquet et al., (1976, 1977) tested serum from BN rats 7-10 days after rejection of a WAG heart graft and found that this serum was more enhancing towards a WAG heart graft than BN anti-WAG lymphoid cells serum, suggesting that an organ specific activity might play a role here. Whether an organ specific component of enhancing sera is really important, or whether the apparent organ specificity reflects differences in the efficacy of immunization, is not known.

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Examples of enhancement across non-Rt1 or non-H-2 barriers have frequently been cited (White and Hildemann, 1968, Goodnight, et al., 1976, Hildemann and Mullen, 1973, Thoenes, et al., 1974). Little more can be said except that grafts across minor histocompatibility differences can be enhanced, and more easily than across major differences (Hildemann and Mullen, 1973). Minor histocompatibility loci, in both rats and mice, are almost totally undefined in terms of their detailed organization; precisely what it is about them that makes them "minor" is not known (Snell et al., 1976).

Enhancement can occur across the strongest histocompatibility barriers. Virtually all the work in organ graft systems agrees that anti-I region activity in enhancing sera is central; in the mouse, there is work narrowing this down to a single subregion within the I region, I-A (Staines et al., 1975a, McKenzie and Henning, 1977). Indications exist in some systems also that activity against just K and D type antigens participates in enhancement, both in skin and heart graft models (Davis, 1977, 1979, and Baldwin et al., 1978) and in one study on tumor grafts (Duc, et al., 1978). It was earlier noted (Section V.3) that enhancement can be achieved across the H-2D end of the mouse H-2 complex (Davis and Ferebee, 1975), which is not known to contain a locus or loci with properties similar to I region loci. Thus any antigens, even organ specific or other minor antigens may potentially

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influence enhancement if antibodies to them are generated. The critical role for I region loci in enhancement may appear because most studies have focused on major histocompatibility differences.

4. Comprehensiveness of Coverage

Any two inbred strains of rats or mice will differ by many more antigens than just those determined by the major histocompatibility complex. Many, if not most, of these genetic differences in cell surface alloantigens are not even defined. Thus it is an important question for the purposes of enhancement whether enhancing antibody needs to include coverage for all of the differences between graft and host or just a critical subset of them.

Moller's (1963a) careful experiments on tumor enhancement suggested that for an H-2 incompatible tumor, the best enhancement was achieved when passively transferred antibody included activity against all the antigens by which the tumor differed from the host. He found that enhancement of the MACD sarcoma (H-2a x H-2f) in C3H mice (H-2K) was most effective when both a C3H anti A (H-2a) serum and a C3H anti-A.CA (H-2f) serum were combined; either alone gave partial enhancement but the tumors regressed.

One reservation about Moller's work is that he defined enhancement quite stringently as progressive tumor growth leading to death of the host. In many of his data-figures, incompletely enhanced tumors show better growth than the

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same tumor in untreated control mice but the incompletely enhanced tumors did not ultimately kill their hosts. It is thus important to note that enhancement is not an all-or-none phenomenon, but can show a continuum of effects.

Amos et al., (1970) studied enhancement of the BP.8 (H-2k) tumor in C57B1 (H-2b) mice given either C57B1 anti-BP.8 or C57.F anti-BP.8 serum. C57.F, congenic with C57B1, will respond to immunization with BP.8 by making fewer antibodies to it since C57.F and BP.8 share several more specificties in the H-2 complex than does C57B1 and BP.8. Using an *in vitro* incubation step followed by *in vivo* tumor growth in C57B1 hosts, Amos, et al., (1970) found that C57.F anti BP.8 serum would enhance this tumor but not as effectively as C57B1 anti BP.8 serum. Consistent with Moller's (1963a) work, "incomplete" antiserum did enhance tumor growth but not as fully as did "complete" antiserum.

Several experiments in organ graft enhancement models suggest that enhancement can follow passive transfer of "incomplete" antisera. Fabre and Morris (1974) enhanced (DA x Lewis)F1 rat kidneys in DA recipients with DA anti-Lewis serum but observed even better enhancement with DA anti-AS2 serum. AS2 kidneys grafted to DA recipients, without enhancement, were rejected; thus DA and AS2 differed by histocompatibility loci (including Rt1; Gunther and Stark, 1977) strong enough for graft rejection. The cross-enhancement observed with anti-DA and anti-AS2 sera might

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have resulted from the sharing of I region specifities; clearly, though, the very existence of this cross-reaction demonstrates that organ graft enhancement can result when not all the antigens of the graft are covered.

Staines, et al., (1975a) obtained similar results studying enhancement of various congenic mouse skin grafts onto B10.D2 (H-2d) or B10.A (H-2a) hosts with either B10.D2 anti-B10.A or B10.A anti-B10.D2 antisera. The grafts presented different degrees of H-2 incompatibility. Their results indicated that enhancement could result when not all the antigenic specificities of the graft were covered e.g. B10.D2 anti-B10.A (specifc for H-2K and H-2I loci) would significantly enhance B10 (H-2b) grafts onto B10.D2, even though B10 grafts present many more incompatibilities than are covered by the anti-B10.A serum. In general, better enhancement resulted as a function of the more specificities included in the antiserum, with a special role found for anti-I-A subregion antibodies.

The final word in this section belongs to McKearn et al., (1979). This group has generated monoclonal hybridoma antibodies, of Lewis anti-BN type; these antibodies are absolutely homogeneous, and consist of exactly one specificity only. Two of their monoclonal antibodies, both IgG, very effectively enhanced (Lew x BN)F1 kidneys grafted to Lewis hosts. Furthermore, since BN RBC were used to screen for these antibodies, their activity is strictly

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against classical serologically defined antigens and not against I region determinants. Thus in this system, in which the antibody is as clean as possible, enhancement of an organ graft follows passive transfer of antibodies to only a single determinant present on the graft.

5. Anti-Receptor Antibodies

Anti-receptor antibodies (ARA) are antibodies with specificity for the combining region of other antibodies or cell surface receptors; by combining with them, ARA are postulated to block the expression of function by these antibodies or receptors (Ramseier and Lindemann, 1975).

One way of generating ARA is to inject parental strain lymphoid cells into an F1 hybrid. The parent cells possess a receptor for the foreign alloantigens of the F1; the F1 does not possess this receptor, since it is tolerant of its own alloantigens, and will thus regard this receptor structure as antigenic and respond to it. This approach has been used by Davies (1971) in mice to generate F1 anti-parental receptor sera which gave some prolongation of skin grafts from the other parent of the F1. Binz and Wigzell (1976) have reported prolongation of rat skin grafts using direct immunization of Lewis recipients with aggregated Lewis anti-DA antibodies. The resulting antibody also specifically blocked Lewis anti-DA mixed lymphocyte cultures.

To date, virtually all reports of ARA activity have been functional. No one has purified them in large enough

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quantities to say to what class or subclass they belong, or really what the "antigen" for which they are specific is since the T cell receptor for alloantigen remains mysterious. Thus, although anti-receptor antibodies may belong in this section on enhancing antibodies, absolutely nothing can be said about them which can be related to the other features of enhancing antibodies discussed above. There are as many reports claiming activity of ARA in different functional assays as there are reporting failure. Due to their diversity, neither methods for generating ARA nor experiments using them are easy to compare. The role ARA may play in organ graft enhancement must still be regarded extremely cautiously until these antibodies are better understood. Nevertheless, as with most other aspects of antibody-mediated enhancement, they may play a partial role in some situations (see Section VII.2).

6. Antibody: An Overview

Antibody is the necessary criterion for enhancement. IgG antibodies and particularly IgG2 antibodies enhance both tumor grafts and organ grafts. The cytophilic and complement fixing Fc portion of the antibody molecule seems necessary. Most enhancement studies involve graft-host combinations which cross the major histocompatibility barrier of the species used. In the mouse, where the organization of the H-2 complex is best defined (though hardly understood), a very important role for antigens determined by the I region has emerged; however, other studies indicate that also, antigens

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determined in the adjacent non-I K and/or D regions can exert an important influence. Organ specific or minor histocompatibility antigens may also be important. The degree of enhancement observed is clearly better, the more target (graft) antigens are covered by the antibody and for a graft bearing numerous strong antigens, such as H-2 antigens in the mouse, the best enhancement results when coverage is total. However, partial enhancement results with partial coverage by antibody and for some tissues or organs, such as the kidney, this may be enough to ensure success; for others, such as skin, partial coverage of graft antigens will barely enhance, if at all. The demonstration of rat kidney enhancement with monoclonal antibody (McKearn, et al., 1979) establishes that for some organs at least, total coverage is not a requirement nor are anti-I region antibodies. A final antibody activity which may be important in some enhanced states is anti-receptor antibodies; however a conclusive demonstration of this remains to be done.

VII. Mechanisms

Two characteristics of enhanced organ grafted animals require explanation: the long term survival of genetically foreign tissue in the face of the clearly demonstrable ability of the host, albeit with various changes in degree, to respond towards donor alloantigens, and the resistance of grafted organs, particularly the kidney in rat and mouse systems, to immunity induced in the enhanced host. In theory, mechanisms of enhancement can act at the level of

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the host immune response, or at the level of the graft either by altering some intrinsic quality of immunogenicity or protecting it from the effects of the host's immune response.

Almost immediately, problems emerge. As has been discussed in earlier sections of this review, enhanced organ graft recipients retain considerable cell mediated immunity in various assays (Section IV. 2.D, IV. 3.D, IV.4). This suggests that enhancement cannot work by significantly abrogating the generation of an anti-graft response and is thus not effected by "afferent" or "central" inhibition. Furthermore enhancement virtually never extends to a donor strain skin graft. Clearly properties of the organ or tissue involved are important. However the quantities of antibody which can enhance in certain systems are so small that peripheral inhibition seems highly unlikely as well (Hutchin, et al., 1967; McKenzie, et al., 1971; Winearls, et al., 1979; de Wall, et al., 1979).

Also, upon regrafting to new, unmodified hosts, enhanced grafts are rejected, suggesting that terminal or efferent inhibition is not effectively protecting the graft from immune responses or preventing the graft from generating them anew. Thus it seems theoretically impossible for enhancement to work at all since everywhere in the immune sequence it might work, there exist good reasons to doubt that it does. Although, in this section, the actions

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of enhancing antibody on the immune responses of grafted animals will be considered, it should be noted at the start that the actual mechanisms simply are not known.

In virtually every review on immunological enhancement, whether for tumors or organ grafts (Kaliss, 1969; Snell, 1970; Voisin, 1971; Russell, 1971; French and Batchelor, 1972; Feldman, 1972; Carpenter, et al., 1976) the discussion of the mechanisms of action of enhancing antibody is divided into three parts. Afferent inhibition covers the prevention by antibody of sensitization of the host immune system to the antigens of the graft. Central inhibition denotes the antibody mediated failure of the host immune system to process otherwise immunogenic material and information from the graft. Efferent inhibition describes the prevention by antibody of effector function by the agents of the host immune response after they have been activated by the graft. Both afferent and efferent inhibition imply a peripheral interaction of antibody with graft antigens, while central inhibition implies a direct action of antibody on the immunocompetant cells of the host.

This division is far too simple, given the enormous complexity of the immune response; the number of points in the sequence of an immune response where antibody can affect its nature or extent is large (Uhr and Moller, 1968; Schwartz, 1971). Thus the division of this process into afferent, central, and efferent is arbitrary and further

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subdivisions can be (and have been) made.

An important point here is that the three classes of inhibition need not be mutually exclusive; there is experimental evidence for each, often for more than one co-existing in a given graft-host system. In addition, antigen:antibody complexes can form at any time after both the graft and the antibody are given; these complexes, rather than the antibody per se, may be causing the observed results. In this discussion of mechanisms, the effects of passively administered antibody and actively induced antibody mediated enhancement will be considered together since there are no compelling reasons to regard them as qualitatively different (Voisin, 1971b). The advantages of studying passive enhancement are that the antibody can be given in defined amounts at predetermined times to try and elucidate the mechanisms of its effect; and also the results are more consistent (Kaliss, 1962). Most mechanistic studies have employed passive enhancement systems.

1. Peripheral Interactions of Antibody with the Graft

The distinction between afferent action by enhancing antibody and central action is impossible to define precisely; primarily this is due to the continued obscurity of many of the events in the development of an immune response. Here, experiments which suggest that the action of enhancing antibody can occur early in the immune response will be discussed although the hesitation must be noted that

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many of these results can as easily be regarded as central interactions of antibody with the immune system. The possible effect of enhancing antibody on passenger stimulatory cells from the graft will also be discussed.

a) Afferent Inhibition

Several lines of evidence point to an action by enhancing antibody early in the sequence of events after a histoincompatible organ or tissue graft is performed. Most commonly in both passive enhancement and combined antigen and antibody mediated enhancement, a single injection of anti-donor antibody is given at or close to the time of grafting (French and Batchelor, 1972; Fabre and Batchelor, 1975; Soullilou et al., 1976; Marquet et al., 1976; Stuart et al., 1976a; Catto et al., 1977; Weiss et al., 1978a; Baldwin et al., 1978; Winearls et al., 1979; McKearn et al., 1979). In a number of earlier studies where the comparison was done, no advantage of multiple injections over a single injection was seen (Jeekel, et al., 1972; Corry et al., 1973a).

Phillips et al., (1973) found that enhancing antibody was very effective at inhibiting the MLR between donor strain stimulator cells and recipient strain responders; the cell mediated lympholysis reaction, representing effector functions, was also sensitive but considerably less so than the recognition reaction of the MLR. Sugarbaker and Chang (1979) confirmed this in vivo, showing that antiserum given

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with a skin graft prolonged its survival, but if the serum was given 10 days after the graft, no enhancement resulted.

In tumor systems, a direct approach to afferent inhibition has been tried. Moller (1963b) pretreated the MACD sarcoma (H-2a x H-2f) in vitro with A.CA anti A (H-2a) serum and injected the tumor into A.CA (H-2f) recipients. He observed enhanced growth of the sarcoma. If he treated the antibody pretreated tumor cells with trypsin, to clear the surface of large proteins, before injection, he observed much less enhancement, suggesting that cell bound antibodies mediated the phenomenon. Moller (1964) divided a C57B1 (H-2b) sarcoma in two parts, pretreated one with A.CA anti-C57B1 antiserum, and injected both parts into an A.CA host. The antibody pretreated tumor grew better than the untreated tumor. Amos et al., (1970) also found that preincubation of the BP.8 tumor in antiserum would, upon injection, allow enhanced growth but that the turnover of antibody off the tumor cell surface, perhaps in the form of complexes, was quite rapid and most of the bound antibody had cleared after 6 hours at 37°C. This dynamic process at the tumor cell surface may explain Kaliss and Rubinstein's (1967) earlier failure to find enhanced growth of the SaI sarcoma after <u>in vitro</u> pretreatment with antibody, although other differences in the tumors used could account for this as well.

Using rat skin grafts, Zimmerman and Feldman (1970) found that immersing an allograft in enhancing serum before

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grafting would prolong its survival and that this prolongation was specific. When a pretreated graft and a normal graft were performed together, only the pretreated one was enhanced, suggesting a peripheral action of the enhancing antibody at the level of the graft itself.

Another feature of enhanced tissue situations often cited as evidence for afferent effects is changes in the kinetics of cell mediated or humoral responsiveness after grafting. These experiments are particularly difficult to distinguish as afferent or central because the observation of a change in the response profile does not permit determination of exactly where or how it came about. Nevertheless, a number of investigators studying tumors or normal tissues, have observed delays in the induction of cell mediated responsiveness (Kalis and Rubinstein, 1967; Peter and Feldman, 1972; Biesecker, et al., 1973; Burgos et al., 1974; Strom et al., 1975). These delays were never more than a few days, and generally, the magnitude of the cell mediated response was barely affected. Burgos et al., (1974) and Strom et al., (1975), both studying passive enhancement of rat kidneys, noted lessened antibody responses after grafting, but antibody was not significantly affected in the earlier studies cited.

Evidence does exist, then, which is consistent with an early action of enhancing antibody on the responsiveness of the host to an allograft. The site of action of this effect,

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in some models at least (Moller 1963b, 1964; Zimmerman and Feldman, 1970) is at the graft itself, in the sense that a systemic alteration in host responsiveness does not occur.

b) Efferent Inhibition

In a number of models, enhancing antibody can be shown to influence the rejection response to an organ or tumor graft when administered many days after grafting, at a time when the host has begun to make a response. That passively administered antibody can enhance tumor growth even when the antibody is given 7 days after the tumor is an old observation (Kaliss, 1962), although it was not determined whether the antibody protected the tumor cells from the host response or interfered at an earlier stage with its development.

Moller (1963c) found that pretreatment of a mouse sarcoma with enhancing antibody afforded it considerable protection when the tumor cells were inoculated back in vivo together with immune lymphoid cells; if the antibody was injected i.p., instead of directly associated with the tumor, it gave less protection from immune destruction. This suggested an effect of the antibody on the tumor cells rather than at an earlier stage on the effector cells.

Using murine in vitro systems, E. Moller (1965), Brunner, et al., (1967), Amos et al., (1970) and Takasugi and Klein (1971) found that antibody to an allogeneic tumor could protect it from destruction mediated by immune spleen

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or lymph node cells. Characteristically in these experiments, the release of ^{51}Cr by the target cells is less in the presence of antibody than in its absence. Whether this is due to competition for surface antigens on the target cells or an earlier action by the antibody (perhaps in complexes with target antigens) on the effector cells is not known from these studies.

Stuart et al., (1971), Biesecker et al., (1973), and Zimmerman (1977) have studied passively and actively enhanced rat kidney recipients with the microcytotoxicity test. All three studies found reactivity in this test against donor strain cells and blocking of this reactivity with serum from enhanced grafted rats. Biesecker et al., (1973) compared the microcytotoxicity test with the ^{51}Cr release assay and found that while enhanced rat cells were positive in the former, they were negative in the latter. In addition, hyperimmune anti-donor serum would inhibit in the microcytotoxicity test; thus the blocking by enhanced rat sera in this test may reflect the presence of anti-donor antibody.

Bowen et al., (1974) passively enhanced (AS x AUG)F1 rat kidneys in AS recipients; they then parabiosed these enhanced AS rats with AS partners hyperimmunized to AUG antigens. The enhanced partners did not reject their grafted kidneys and the responsiveness of the hyperimmunized partner to AUG antigens was noticeably lessened, suggesting that

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there was something "dominant" about the enhanced condition in this model. Russell et al., (1978b) in their spontaneously long surviving mouse kidney graft model (Section IV.5) found that the adoptive transfer of immune lymphoid cells into the grafted recipient caused transient but not extensive damage to the grafted organ, also indicating that some protection of the graft was present.

If efferent inhibition was a major component of the success of histoincompatible grafts, such grafts might be expected to survive well in presensitized recipients. Generally this is not the case (Jeekel et al., 1972; Marquet et al., 1977b) although it has been claimed (Fabre and Morris, 1975) that presensitization can be overcome with enhancing antibody. The immune rejection of a donor strain skin graft does not lead to the rejection of the enhanced kidney (Fabre and Morris, 1975). It is possible that in some of these experiments, the borderline between presensitization and the induction of active enhancement has been obscured.

One of the most perplexing experiments in enhancement was done by Kaliss (1962). He gave SaI to an allogeneic mouse, without enhancement. 7 days later, he gave a second injection of SaI, this time with enhancing antibody. The first dose of SaI underwent enhancement and grew progressively; the second dose of SaI was immunologically destroyed. Thus in the same animal, enhancement and a second

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set rejection response directed to the same tissue can occur simultaneously. Antigenic change in the first SaI graft was excluded as a possible explanation. Whatever effect the injection of antibody had in this experiment could not have been central because then it should have been systemic; both the first and the second tumor should have been either enhanced or destroyed. A peripheral effect would be expected to act to protect the second tumor as well rather than just the first one. What the experiment serves to demonstrate is the complexity of the enhancement phenomenon.

c) Graft Alterations

One possible way in which a graft might avoid the full force of a rejection response is to alter the appearance it presents to the host immune system. Precedents for such "immunologic modification" exist in several systems (reviewed in Jacobs and Uphoff, 1974). The essential test for such graft adaptation is retransplantation of the grafted tissue to a new recipient syngeneic to the former recipient; if the graft is now not rejected as quickly as a fresh graft of identical type, it is concluded that adaptation has occurred in the first graft due to its residence in the former recipient.

McKenzie et al., (1972) and Nirmul et al., (1972) grafted allogeneic skin to nonspecifically immunosuppressed (ALS or irradiation) and specifically enhanced (McKenzie, et al., 1972) mice. Two weeks later, a second skin graft from

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the same donor strain was performed. The second skin graft was rejected normally while the first graft showed prolonged survival. This greater susceptibility of a fresh graft over an "enhanced" graft was interpreted to be partly due to graft adaptation; possible effects of the immunosuppression, however, could also influence the rate of recovery of allograft immunity as well. Superficially, these experiments resemble Kaliss' (1962) cited above, in which, however, graft alteration was excluded.

Warden et al., (1973) grafted DA rat thyroids into the anterior chambers of the eyes of thyroidectomized Fischer recipients. After 3 months in the anterior chamber, the thyroids were grafted subcutaneously in the same recipients and found to function well. If grafted to a freshly thyroidectomized Fischer rat, the "in vivo cultured" thyroid was rejected, suggesting that in this model some alteration in both the graft and the host may have occurred.

Recently, using a xenogeneic model of rat heart and skin grafts to initially ALS-immunosuppressed mice, Burdick et al., (1979) have found that long term surviving rat heart xenografts are highly susceptible to passively transferred mouse anti-rat antibody but that rat skin xenografts, resident for a similar length of time (about 40 days), are often completely resistant to this antibody. The suggested reason is the replacement of vascular endothelial cells in the skin graft by host endothelium, a process not thought to

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occur in the heart graft. Whether an analogous process can occur in allografts is not known.

While some degree of graft alteration in surface antigenicity may occur, it is known that certain components of antigenicity do not change. Using ^{125}I-labeled anti-donor antibody, Fine et al., (1973) found that enhanced rat kidneys absorbed as much of this antibody as did freshly grafted non-enhanced kidneys. Mullen et al., (1973b) did analagous experiments, also in rats, and found that ^{131}I-anti-donor antibodies rapidly disappeared from the circulation of enhanced kidney grafted rats. French (1973) measured the disappearance of cytotoxic anti-donor antibody from the circulation of long standing enhanced kidney grafted rats and found that the remaining cytotoxic titer was decreased. All of these experiments indicate that at least some antigens, determined by the rat Rt1.A complex, remain exposed on enhanced kidney grafts, and further suggest that the primary mechanism of enhancement in these models may not be efferent immunoprotection. The one contradictory report is of Morris and Lucas (1971) who claimed that passively enhanced rat kidney grafts did not bind ^{125}I-anti-donor antibody. This was interpreted to be due to peripheral coating of antigens on the grafted kidney by enhancing antibodies.

d) Passenger Cells

Another way a graft might present an altered appearance

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to the host immune system is if certain components which are especially immunostimulatory are removed before they can interact with host lymphocytes. Graft components thought to be the most potent for this role are donor type lymphoid cells trapped in the spaces of the graft when it is transplanted. (Snell, 1957; Billingham, 1971) A corollary of this idea is that immune responses have special requirements for initiation but that for effector action, any tissue bearing foreign alloantigens can be attacked (Lafferty and Cunningham, 1975; Lafferty and Woolnough, 1977).

The role of passenger cells has been investigated both by removing them to see what influence this has on graft fate and also by replacing them with passenger cells of different genotype. Halasz and Orloff (1965) pretreated vascularly isolated donor rabbit ears with whole plasma from a presensitized rabbit and found a slight delay in rejection of skin from the treated ear over untreated skin. Stuart et al., (1971) irradiated F1 rat donors prior to kidney grafting and found no significant delay in the onset of rejection of irradiated F1 kidneys over normal F1 kidneys when grafted to a parental recipient. Kyger and Salyer (1973) irradiated Lewis rat donors of skin grafts to Buf recipients and observed a slight prolongation over unirradiated donor skin. Both Schanzer et al., (1974) and White et al., (1977) found with F1 to parent rat kidney grafts that the longer the donor kidney was without circulation after removal from the donor and before vascular

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connection with the recipient, the better the fate of the graft, suggesting that some component of the graft which was highly vulnerable to ischemia was important for graft immunogenicity. Other attempts at removal of passenger cells with anti-lymphocyte globulin (Nelson et al., 1975, in a rat heart grafting model) or by perfusion with medium (Chartrand and Stanley, 1979, in a dog heart grafting system) have given opposite results; no prolongation was achieved in the rat model while significant prolongation was achieved in the dog model.

The best worked out system for the removal of passenger cells is that of Lafferty and colleagues, using a period of in vitro culture to deplete thyroid grafts of stimulatory cells (Lafferty et al., 1975; Lafferty et al., 1976; Talmage et al., 1976; and Lafferty and Woolnough, 1977). In this model, organ culture for three weeks removes immunogenicity from mouse thyroid tissue allowing transplantation across H-2 barriers into thyroidectomized but otherwise unmodified recipients. If peritoneal exudate cells (PEC) are grafted with the cultured thyroid, it is rejected indicating that the thyroid is still susceptible to rejection but that the culture period depletes it of an ability, restorable with PEC, to initiate an allograft response (Talmage et al., 1976). Lacy et al., (1979) achieved a similar prolongation of rat pancreatic islet cells by in vitro culturing.

A number of studies have attempted to evaluate the

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importance of passenger cells versus structural graft parenchymal cells by replacing the initial passenger cells with new ones. To this end, Guttmann and Lindquist (1971) made bone marrow chimeras of (Lew x BN)F1 rats by injection of cyclophosphamide and Buf strain cells. Kidneys from these rats, which were (Lew x BN)F1 structurally but of Buf genotype hemopoietically, were grafted to Lewis recipients sensitized either to BN antigens or Buf antigens. In the former case, kidney rejection resulted, in the latter, enhancement resulted. Thus the immune response, here representing active enhancement, against the passenger cells led to enhancement. If Lewis bone marrow was infused into a (Lew x BN)F1, and then kidneys from this chimera grafted to a normal Lewis host, there was no acute rejection of the kidney; this illustrated that allogeneic passenger cells can induce a strong rejection response when present, a result found in other situations as well (Elkins and Guttmann, 1968; Barker and Billingham, 1972).

That passenger cells can stimulate strong immune reactions is a repeated observation (Billingham, 1971); however, a number of experiments have indicated that a passenger-cell-depleted allograft can remain immunogenic (Stuart et al., 1971; Kyger and Salyer, 1973; Nelson et al., 1975). Certainly a long standing enhanced F1 organ graft can be rejected if retransplanted to a sensitized secondary recipient (Batchelor et al., 1979) and retransplantation experiments, cited earlier (Stuart et al., 1969; Fabre and

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Morris; 1972b; Jenkins and Woodruff, 1973) indicate that an unsensitized secondary recipient will also reject previously enhanced organs. Thus passenger cells cannot be the only source of immunogenicity in an organ graft, although they may certainly be an important source. At least two studies (Nelson et al., 1975; and Welsh et al., 1979) indicate that the major immunogenicity of a rat kidney does not disappear rapidly upon grafting but that more than 72 hours are required; whether this reflects the time required for graft passenger cells to traffic out of the organ, or for some other physiologic alteration to take place is not known.

The exact contribution of passenger cells to the stimulation of an immune response is the subject of much research, but this contribution remains obscure. Ultraviolet radiation destroys the stimulatory ability of lymphoid cells (Lafferty et al., 1974) while a factor obtained from supernatants of concanavalin-A stimulated spleen cells can nonspecifically restore it (Talmage et al., 1977). The role of Ia antigens as the source of passenger cell immunogenicity is also uncertain; cells not known to express Ia antigens, such as platelets and RBC, do not stimulate antibody in vivo (Welsh, et al., 1977; Batchelor, et al., 1978) nor active enhancement (Jenkins and Woodruff, 1971; Gallico and Mason, 1978). If the expression of Ia is of critical importance, it need not come from passenger cells; rat kidney parenchyma is reported to be Ia positive (cited in Morris, 1979).

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e) Peripheral Inhibition: An Overview

Enhancing antibody can interfere with the development of a host immune response by acting on the graft at the initiation of a response, or after a response to the graft has developed. Antibody passively transferred at grafting, but not several days later, can induce enhancement of organ grafts; grafts of tumor tissue may have different requirements since antibody given up to 7 days after grafting can enhance (Kaliss, 1962). Repeated injections of antibody usually do not benefit organ grafts. Preincubation of tumor or skin grafts in enhancing sera prior to grafting can result in prolongation of survival and this effect is specific to pretreated grafts, not to identical but untreated grafts transplanted simultaneously. Frequently, the development of cell mediated immunity to an enhanced graft is delayed, but never for very long. Both in vitro and in vivo, enhancing antibody or the enhanced recipient can give some protection of tissue or tumor grafts from a developed host immune response. However, as Kaliss' (1962) experiment demonstrates, enhancing antibody can act in unexpected ways not easily characterized as peripheral or central. Surface changes in enhanced grafts can occur, rendering them less visible or susceptible to a host response. Exactly what the nature of these changes may be remains unclear, however, because other work indicates that major histocompatibility complex antigens can remain exposed on the graft. Finally, passenger stimulatory cells, present

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in the graft when it is transplanted, can add appreciably to its immunogenicity; one effect of enhancing antibody may be to mask or clear these cells from the graft. Just what feature or activity these passenger cells contribute to graft immunogenicity is far from clear.

Much of the controversy in the literature on enhancement derives from the expectation that if antibody acts at one particular stage in the immune response, it should not be expected to act at another stage in the same immune response. With the possible exception of Moller's work (1963 a,b,c), the action of enhancing antibody at more than one point in a response has not been investigated. As noted before, the immune response, from induction through development to the effector stage, is highly complex and can be influenced at many points. Given the multiplicity of different models which have been studied, it should hardly be surprising that so many different effects of antibody, frequently described in ways that appear mutually exclusive, have been reported.

2. Central Inhibition

This is the hardest "mechanism" to define. Furthermore, a number of investigators have concluded that, in their systems, enhancing antibody acts centrally because they could not demonstrate obvious afferent or efferent effects; thus the conclusion of central inhibition was arrived at by elimination. (Brent and Medawar, 1962; Brondz, 1965)

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a) Evidence

The type of positive evidence that can be cited for central inhibition includes the often minute amounts of antibody which can enhance, the fact that most enhancing antibodies are of the IgG class (see Section VI), and evidence of depressed cell mediated reactivity of lymphoid cells from enhanced animals. The prominence of Ia antigens on a graft as targets for enhancing antibody can be cited. Analogies are drawn between enhancement and antibody interference with other immunological reactions. Also observed in a few models is the stable maintenance of enhancement and its extension to other tissues with time after establishment. Reports suggesting that suppressor cells or unusual antigen processing are involved in enhancement also exist.

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One complication in interpreting many enhancement experiments is that enhancing antibodies can persist for considerable lengths of time after passive transfer (Rubinstein and Kaliss, 1964). The effect ultimately being measured may not be induced by the antibody when it is first given but occurs later when antigen is administered and complexes can form. Whether resulting inhibition is described as afferent or central can become a semantic debate.

That small amounts of antibody can enhance large grafts of tissue has been noted before; suffice it to say here that

these amounts of antibody do not protect a graft from a rejection response and seem insufficient to mask the presence of a graft from the entire host immune system. As noted above, this rationalization for a central action of antibody is weak and, taken by itself, unconvincing.

That enhancing antibodies are generally IgG is consistent with the known immunoregulatory properties of IgG antibodies in other systems (Uhr and Moller, 1968; Schwartz, 1971; Rowley et al., 1973; Fitch, 1975). The known diversity of IgG subclasses, with different properties and functions (Spiegelberg, 1974; Winkelhake, 1978) makes them appealing candidates for immunoregulators in vivo, but, as evidence, is hardly decisive.

A reduced reactivity towards graft allo-antigens by host lymphoid cells is noted in a number of studies. Typically, lymphoid cells from an enhanced host are mixed with appropriate tumor cells and together injected into a host syngeneic with the source of the lymphoid cells. The subsequent growth of the tumor cells is used as an assay for the activity of the lymphoid cells.

Using a related approach, Mitchison and Dube (1955) found reduced reactivity in lymph node cells from mice actively enhanced to, and grafted with, Sa1 sarcoma measured by the growth of the tumor in vivo. A concern expressed by these authors was that antibody producing cells may have been transferred with their lymph node preparation and this

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may have accounted for the enhanced tumor growth. A similar reservation may be expressed about the results of other experiments of this kind.

Snell et al., (1960), Takasugi and Hildemann (1969b), and McKenzie et al., (1971) all described lessened immune reactivity in cells from antibody pretreated mice. Snell et al., (1960) determined that mice given antibody and allogeneic normal tissues also showed this depressed reactivity so it did not follow from tumor grafting only. Takasugi and Hildemann (1969b) measured the change in peripheral lymphocyte numbers after enhancement and found no increase, in marked contrast to the lymphocytosis which followed immunization with tumor. Futhermore, this effect of enhancing antibody persisted after removal of the tumor mass itself, directly suggesting a central site of action since the assumed site of any peripheral action, the tumor, was gone. Studying enhancement of skin grafts, McKenzie et al., (1971) found a sharp decrease in the reactivity of cells from the lymph node draining the graft site in enhanced mice. All of these studies concluded that antibody acted centrally to lower anti-graft immune reactivity.

Mitchell (1972) gave antibody to mice shortly before leukemia injection, and then measured the cytotoxic response of spleen cells from these mice against tumor strain targets, finding a significant reduction in reactivity. Antibody given after the tumor did not reduce spleen cell

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mediated cytotoxicity in vitro. Adoptive transfers of antibody pretreated spleen cells also resulted in decreased reactivity to the tumor, suggesting a central rather than peripheral action of this antibody on the immune response. This likely was induced at the time the tumor immunogen was added, since, as noted earlier, enhancing antibody persists in vivo (Rubinstein and Kaliss, 1964).

The prominence of I region antigens in enhancement has been discussed (Section VI.3). That this may suggest a central action for enhancing antibody derives from the role I region antigens are thought to play in the induction of a wide range of immune reactions (Klein and Hauptfeld, 1976; McDevitt et al., 1976) Thus if anti-I region antibodies in enhancing sera combined with graft Ia antigens and exerted an effect on responding lymphocytes preventing them from being triggered (see Meo et al., 1975; Kano et al., 1976; Schwartz et al., 1976 for in vitro evidence for this), an antigraft response could be prevented.

A number of reviewers (Kaliss, 1970; Snell, 1970; Rowley et al., 1973) have drawn parallels between antibody mediated allograft enhancement and antibody mediated suppression of responses to sheep red blood cells (SRBC). Minute amounts of antibody are effective in both systems (Haughton and Nash, 1969). The immunosuppression induced by anti-SRBC is both specific and persistent (Haughton and Nash, 1969; Ryder and Schwartz, 1969). In a careful study of

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the effects of anti-SRBC antibody on the subsequent response to this antigen, Ryder and Schwartz determined the lesion in the suppressed mice to be in the macrophages, a result consistent with central inhibition of this response. Both of these studies focused on the antibody response rather than a cell mediated response; however, reports of antibody mediated suppression of cell mediated responses have also appeared (Axelrad and Rowley, 1968; other citations in Fitch, 1975).

In several organ graft enhancement models, it has been reported that, with increasing time after the induction of enhancement, it becomes progressively easier to successfully graft different donor strain tissues, culminating in that most difficult graft, skin. Linder (1962) subsequent to an ovarian graft, and Mullen et al. (1973a), Thoenes (1975), and Poole et al., (1976) subsequent to rat kidney grafts, all reported eventual success with donor strain skin grafts or, in the case of Poole et al., (1976) entire rat hind leg grafts. It might be concluded from these reports that with time after enhancement induction, a maintenance phase sets in during which almost any donor strain tissue may be grafted without rejection. This maintenance phase would be mediated by the presence of an activity since enhanced animals are resistant to the breaking of the enhanced condition (Batchelor et al., 1977). However in many other reports, in which enhancement of an organ graft has been established for extended periods of time, it was found that

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no lessening of the response to donor strain alloantigens, especially when presented on a skin graft, resulted (Stuart et al., 1969; Jenkins and Woodruff, 1971; Fabre and Morris, 1972b; Desi and Ruszkiewicz, 1977). This unexplained discrepancy is yet another of the many contradictory features reported about enhancement.

Yet another way that central inhibition may be achieved is by the intervention of anti-receptor antibodies (ARA, section VI.5). As noted previously, ARA have been assayed by their effects on in vitro and in vivo tests; as yet no easy and reliable direct assay for them exists. Their existence and action have been inferred in a number of experimental systems of organ grafting and graft-versus-host disease.

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When live lymphocytes from one strain of rat or mouse are given to another strain of rat or mouse under circumstances in which the immune system of the recipient is unable to reject the donor lymphocytes (an F1 hybrid recipient, a very young immunoincompetant host, or an immunosuppressed host), the donor lymphocytes attack and damage it (Grebe and Streilein, 1976). Several groups have observed that in F1 hybrid rats recovering from a sublethal GVH reaction, a second normally fully effective injection of parental lymphoid cells fails entirely to induce a GVH reaction (Field et al., 1967; Elves, 1973; Woodland and Wilson, 1977). Consequent to the first incomplete GVH reaction, the F1 hybrid host has become resistant to a

second GVH reaction.

Using serum from such a recovered F1 hybrid, Joller (1972), McKearn et al., (1973), McKearn (1974), Binz (1975), Cauchi and Dawson, (1975), and Kinsky et al., (1978) have all reported that upon passive transfer of this serum to F1 hybrid recipients of parental cells, the resulting GVH reaction was considerably diminished. The globulin fraction of the serum contained this activity (Cauchi and Dawson, 1975) further suggesting that it was due to an antibody.

These antibodies are thought to be directed against the recognition structures by which parental lymphocytes react to F1 alloantigens. Such antibodies are postulated to prevent parental anti-F1 responses by combining with the cell surface recognition structures (Ramseier and Lindemann 1975; Binz and Wigzell, 1976).

Not all reports of work with ARA are positive. Using ARA from Ramseier's laboratory, Lindahl (1972) was unable to block the mixed lymphocyte reaction; Fitch and Ramseier (1976) were unable to block the <u>in vitro</u> generation of cytolytic T-lymphocytes. Kinsky et al., (1978), although able to inhibit an appropriate GVH reaction with their ARA preparation, could not enhance the growth of a tumor of appropriate genotype. While Lucas and Enomoto (1973) reported (in an abstract) that an ARA enhanced rat kidney grafts, others have had difficulty confirming this (Carpenter et al., 1976).

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Voisin (1971a, b) has suggested that classical alloantiserum can enhance in the GVH situation. Here, the antibodies act not by enhancing a graft from the immune response of the host but by enhancing the host from the immune response of the grafted cells. Several groups have found that passively transferred alloantibody can specifically protect an F1 hybrid against parental GVH inducing lymphoid cells (Batchelor and Howard, 1965; Voisin et al., 1968; Voisin, 1972b; Fink et al., 1974). Thus A anti-B antiserum given to an (AxB)F1 along with A lymphoid cells reduced or prevented a GVH reaction but had no effect on the GVH reaction caused by B strain cells.

Two papers investigating GVH reactions noted a peculiar result which will be discussed later in RESULTS. Fox (1962) induced GVH reactions in chromosomally marked adult (CBA/T6xC57B1)F1 hybrids with 10^8 C57B1 spleen cells. Upon chromosome analysis of the cells in the spleen of the recovered F1 hybrids, he found that all the cells in the spleen, after 60 days, were of donor genotype. In another strain combination, Batchelor and Howard (1965) also found that a large injection of parental spleen cells could lead to a complete takeover of the circulating red blood cells of the F1 hybrid. In this case, the F1 was protected or enhanced by parent anti-F1 serum at the time of cell injection. One effect this serum may have had was to alter the presentation of the F1 circulating lymphoid cells, of

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known stimulatory potency (see Section VII.1.D), to the donor lymphoid cells; exactly how the takeover of the F1 hemopoietic system was achieved was not determined.

With respect to anti-receptor antibodies especially, firm conclusions are difficult to reach. That the graft-versus-host reaction is unusually susceptible to amelioration by preparations believed to have ARA, as well as by more conventional alloantibody, seems established.

For organ graft enhancement, a necessary question is where ARA would come from: passively transferred parental anti-F1 antibody enhances, as does a hybridoma alloantibody, neither of which will contain any ARA (McKearn et al., 1979). For ARA to be present, either cells in the F1 organ graft must produce it (and whether an F1 kidney has enough passenger cells and of the right specificities to do this is not known) or the ARA must be produced by the host as a normal immunoregulatory component (Jerne, 1974; Richter, 1975), but in time to protect the organ graft rather than preventing an excess response to it after it is already doomed. Just how important ARA are in enhancement is very hard to judge.

There are two models, both involving enhancement of rat heart allografts, in which a suppressor cell is reported to participate (Tilney et al., 1978; Hendry et al., 1977, a,b; Hall, et al., 1979). In one system, thymocytes obtained from the enhanced graft recipient are claimed to adoptively

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transfer a degree of specific unresponsiveness, but these thymocytes must be obtained at a very specific time after enhancement and grafting, else they have no effect (Hendry et al., 1979b). In the other model, lymph node cells from enhanced grafted rats are claimed to be able to delay restoration of allograft responsiveness in irradiated recipients but the effect works only at certain effector cell/inhibitory cell ratios. In both models, a suppressor cell may play some role, but it is difficult to conclude from these studies that the role of such a cell is a major one.

Other ways enhancing antibody may interfere centrally with the development of immune responses have been hypothesized. One suggestion is that antibody interferes with helper T-cell function, preventing them from inducing graft-destroying effector cells (Batchelor and Welsh, 1976; Batchelor, 1978). Another suggestion is that antibody, complexed with graft antigen and perhaps affixed to macrophages, interacts with antigen reactive cells and induces their opsonization by the associated macrophages. (Hutchinson and Zola, 1977 a,b; 1979) The first hypothesis does not predict any loss of anti-graft reactive cells, merely their failure, mediated by antibody, to become activated; the second hypothesis does predict the eventual elimination of anti-graft reactive cells which would result in a state indistinguishable from tolerance. There is enough evidence in the enhancement literature to sustain either

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hypothesis, although not to firmly establish either one.

b) Central Inhibition: An Overview

That antibody can act centrally to impair an anti-tumor or anti-graft immune response is supported most effectively by the studies showing a reduced ability of lymphoid cells from enhanced animals to respond against graft antigens. The reservation noted by Mitchison and Dube (1955) must be noted again. These experiments are done by measuring in vivo growth of the test tumor in the presence of lymphoid cells from the pretreated host. If antibody forming cells are part of the cell preparation, then enhanced growth of the tumor might result which could be incorrectly interpreted as lessened cell mediated reactivity. The inhibitory activity of cell preparations carefully depleted of antibody secreting cells has not been checked.

Much of the considerable difficulty separating afferent from central impairment derives from the fact that since this impairment is graft specific, it cannot properly be induced before the tissue in question has been grafted. Once grafting is done, and there is antigen in the system, the possibility exists of forming antigen-antibody complexes, which can travel via the bloodstream and lymphatics throughout the host. Whether the critical interactions of antigen and antibody with the cells of the host then occur at or near the graft, or further away becomes difficult to determine.

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Other ways in which antibody might act include preferentially acting on antigens which play an important role in lymphoid cell stimulation such as Ia antigens, or antigens on helper T cells. Antibodies with activity against parental anti-F1 receptor structures may play a role in enhancement although the evidence at this point is neither direct nor anything close to conclusive. A suppressor cell may be induced although the evidence for this is not strong. Processing of antigens, or expression of them on macrophages may be altered, or conditions might occur in which antigen reactive cells attempting to bind antigen are diverted and perhaps phagocytosed.

The strongest direct evidence that antibody can act centrally to modify an immune response is the work of Ryder and Schwartz (1969) showing that macrophages from antibody suppressed mice are unable to present antigen to otherwise responsive lymphoid cells. Interpretation of this work as pertinent to organ graft enhancement depends upon what similarities exist between the response to sheep erythrocytes, studied by Ryder and Schwartz, and the response to an organ graft. While antibody can regulate the subsequent antibody response to SRBC, the problem confronted by the recipient of an organ graft is to regulate the cell mediated response to its alloantigens. Comparisons of such diverse types of work on immunoregulation must be made with great caution.

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VIII. Parabiosis and Project Rationale

Prolonged survival of foreign tissue can be achieved in adult animals by prior exposure of the recipient to donor type antigens (active enhancement), passive transfer of anti-donor antibody (passive enhancement), and sometimes both procedures combined. Another technique which has been used to induce a measure of unresponsiveness to tissue alloantigens is the physical joining of two entire animals in parabiosis; this exposes the immune system of each partner to very large amounts of antigen from the other (Nisbet, 1973; Finnerty, 1952). When two animals (commonly mice or rats) are joined in parabiosis, cross exchange between the circulatory systems of the two partners is established, allowing antigen exchange. Then a curious and not yet fully understood hemodynamic alteration, namely polycythemia in one partner and anemia in the other, often begins. After 7-10 days, the effects of immunogenetic differences, in those pairs which incorporate antigenic differences, especially at H-2 or Rt1.A, become prominent. Most commonly, the death of one or both partners, or the complete biological isolation of each partner from the other, intervenes by 2 to 4 weeks after joining.

In those strain combinations in which some fraction of the pairs survives, a new set of sequelae is seen. Frequently, cells from one partner can colonize the spleen of the other partner. This can happen in parent-plus-F1-

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hybrid combinations and can vary considerably with the strains involved. Finally, a number of reports (Rubin, 1959; Martinez, et al., 1960a, b, 1961; McBride and Simonson, 1965) indicate that unresponsiveness to alloantigens of one partner can be induced in the immune system of the other partner and a few reports even raise the spectre of immunological enhancement to partly account for this (Rubin, 1959; McBride and Simonson, 1965).

The cross exchange between partner circulations in parabiosis is established quickly. Using isotope labeled red blood cells and serum proteins, Bichel and Holm-Jensen (1949), Binhammer and Hull (1962) and Sodicoff and Binhammer (1964) determined that in syngeneic rat and mouse parabionts, cross circulation was detectable as early as 2 days after parabiosis, increasing thereafter to unhindered values. In non-syngeneic pairs, cross circulation was established just as quickly but never enjoyed the unimpeded flow seen in syngeneic pairs (McBride and Simonsen, 1965). Others had earlier shown that cross circulation extended to circulating leukemia cells (Furth et al., 1940) and to anti-tumor resistance, presumably antibodies (Harris, 1943; Falls and Kirschbaum, 1953). Thus once cross exchange was established, both cellular and humoral traffic was allowed, at least for a while.

A consequence of this exchange, often seen with parabionts incompatible for H-2 or Rt1.A antigens, is a

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polycythemia in one partner and anemia in the other. This syndrome is most often noted when a parental strain partner is parabiosed with an H-2 semi-incompatible F1 hybrid (of the type A plus (AxB)F1 where A and B are two strains differing at H-2); the parental partner most often becomes polycythemic, the F1 partner anemic. In this genetic arrangement, the parental partner treats the F1 as a foreign graft and responds to it; the F1 cannot respond back. The anemia can become more severe with time until the F1 dies. Numerous studies have suggested explanations ranging from immune hemolysis of F1 RBC (Chute and Sommers, 1952) to some variety of selective trapping of F1 RBC in the parental partner, with these cells for some reason being unable to return (MacGillivray and Tokuda, 1962; Tokuda and MacGillivray, 1962). Still others have suggested that immunology is not initially involved at all, that the anemia-polycythemia reflects some intrinsic physiological process (Eichwald et al., 1960, 1961; Hilgard et al., 1963, 1964); only after 1-2 weeks of parabiosis, after an immune response has begun, does the anemia-polycythemia reflect immune processes. What all reports agree on is that somehow there is unequal distribution of the total blood mass within a pair of parent-plus-F1 hybrid parabionts, preferentially to the parental strain partner. That immunological differences become important after 1-2 weeks is not disputed; it is the importance of these differences initially that has been controversial (Hall and Hall, 1956;

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1957).

A more obvious consequence of the parabiosis of genetically incompatible parent and F1 hybrid animals is a complex syndrome called "parabiosis intoxication" (Nisbet, 1973). Most often it is the F1 partner that falls victim to this process, dying in 2-4 weeks, with some variation depending on the strains involved. The exact relationship of the anemia-polycythemia to this intoxication is not clear; anemia-polycythemia can occur in the absence of H-2 differences and the presence of an H-2 difference does not guarantee anemia-polycythemia (Eichwald et al., 1963). The artificial "cure" of the F1 anemia with transfusions of whole F1 blood does not significantly reduce the mortality due to parabiosis intoxication (Cornelius and Martinez, 1965).

The pathology of parabiosis intoxication is characteristic. Besides the F1 anemia and parental polycythemia, there is in the F1 partner a lymphocytopenia, granulocytopenia, reduction in marrow cellularity, thymic atrophy, occasional liver, kidney, and skin lesions, moderate splenomegaly, and curiously an increased reticulocyte count (Cornelius et al., 1967; Cornelius et al., 1968; Kitamura et al., 1969; Jurin and Allegretti, 1970). The polycythemic parental partner, in contrast to the F1, does not show lymphocytopenia or marrow hypocellularity; it does show the splenomegaly (Kitamura et al., 1969).

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What this intoxication closely resembles, as was first suggested by Billingham (1959) on gross morphological grounds, is a graft-versus-host reaction, derived from the recognition of the foreign alloantigens of the F1 partner by the parent partner. That the joining of presensitized parental mice to F1 partners of appropriate target genotype can result in a quicker and more severe intoxication strengthens this interpretation (McBride et al., 1967; Cornelius, 1968; Jurin and Allegretti, 1970). Also, intoxication is not seen in syngeneic pairs.

Since Finnerty and Panos (1951) observed that parabionts of littermate rats frequently never developed parabiosis intoxication as judged by polycythemia-anemia, whereas non-littermate rat parabionts usually did develop it, the role of immunogenetic differences has received much attention. Much work has consistently shown that the weaker the immunogenetic differences incorporated into the parabiosis, the less severe the intoxication and, often, the longer the parabionts will live. This is strongly reminiscent of Hildemann's generalization (Hildemann and Mullen, 1973, see Section V.2) on kidney graft enhancement. H-2 differences play the most important role (Eichwald et al., 1959; Martinez, 1960a; Leonard, 1964; McBride et al., 1967; Nisbet and Edwards, 1973). Also reminiscent of enhancement is that, judged by prolonged survival, a fully allogeneic graft or parabiont partner is less successful

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than an F1 hybrid graft or partner (Eichwald et al., 1959 and see Section V.2). Strain asymmetries exist in parabiosis as well (Martinez et al., 1960a; Nisbet and Edwards, 1973, and see Section V.3) with one parent responding less strongly to a given F1 hybrid partner than the other parent of that F1.

The objective of several studies using parabiosis of adult parent strain and adult F1 hybrid mice has been to induce immunological tolerance, in the parental strain partner, to the alloantigens of the F1 partner. Rubin (1959) reported in one strain combination that unresponsiveness resulted in 40% of his parabionts. Martinez, et. al., (1960a, b, 1961) were able to induce permanent skin graft unresponsiveness by parent-F1 parabiosis; the less the incompatibility between parent and F1, the more successful were the eventual F1 skin grafts to the parental partner. They also found that the parabiosis had to be maintained for a certain minimum length of time (generally 30 days) for "tolerance" to be induced (Martinez et al., 1960b). The mechanism of this unresponsiveness was never ascertained; it appeared to be transferrable with spleen cells (Martinez, et. al., 1961, also Nisbet, 1971) to neonatal mice syngeneic with the parental parabiont. However the methodology of these experiments did not exclude anti-F1 antibody-forming cells from being in the transferred inoculum. Finally, the antigenic disparity between parent and F1 was not a full H-2 difference, only an H-2D end difference, known not to be as

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strong (Section V.3; McKenzie and Snell, 1973; Nisbet and Edwards, 1973). That immunological enhancement might have played a role in several unresponsive states induced by parent-F1 hybrid parabiosis, and one Rt1.A compatible rat vascular cross-circulation system (DiMarco et al., 1971) in which skin graft prolongation was induced, was advanced by a number of authors (Rubin, 1959; McBride and Simonsen, 1965; Nisbet, 1973).

It is repeatedly observed, in F1 mice suffering from parabiosis intoxication, that a high proportion of the cells in the F1 spleen are parental in origin. The majority of these studies (Nakic et al., 1966; Nisbet, 1967, 1971) utilize the T6 chromosome marker; this marker is detectable in dividing cells only and thus these studies can only detect dividing T6-marked donor cells in the F1 spleen or peripheral blood (Nisbet, 1971). McBride and Simonsen (1965) used direct cytotoxic tests with appropriate antisera and complement to show that after 3 to 4 weeks of parabiosis, only parental strain cells were present in the F1 spleen. This takeover was noted before in two graft-versus-host models (Fox, 1962; Batchelor and Howard, 1965). Although Nakic, et. al., (1961) had noted that such a takeover of the F1 spleen by parental cells was theoretically possible, these experiments were the first demonstration of it.

What causes the occasional unresponsiveness seen in parabiosed parent-F1 mice? The possibilities considered

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range from enhancement (Rubin, 1959; McBride and Simonsen, 1965) to classical tolerance (Martinez et al., 1960b, 1961), to sheer antigenic overload (Nisbet, 1973). Another possibility is a suppressor cell (Sloane et al., 1976); the studies in which unresponsiveness was transferred from parental partners to neonatal syngeneic mice with spleen cells would seem initially to support this (Martinez, et. al., 1961; Nisbet, 1971). Only one study, that of McBride and Simonsen (1965), analyzed whether parabionts had antibody. They found that in all of their C3H (H-2k) plus (C3H x ST/A)F1 (H-2k x H-2b) parabionts, both the parental and F1 partners had anti-H-2b antibody; this antibody was hemagglutinating, present in high titers long after parabiosis had been done, (up to 120 days) but was not cytotoxic for ST/A target cells. In this study 65% of the F1 hybrid partners had died by 5 weeks after parabiosis and subsequent mortality was not mentioned. If this antibody was important to the survival of these parabionts, then similarities between them and "classically" enhanced mice would be important to look for. Yet another possibility that can be suggested, in light of the takeover, is that the F1 partner is "cleansed" of immunostimulatory passenger lymphocytes.

Rubin (1959) had reported a 40% long term survival rate for DBA/2J (H-2d) plus (DBA/2J x C3H/HeJ)F1 (H-2d x H-2k) parabionts. Furthermore, the parental partner became tolerant of C3H/HeJ or F1 skin grafts but there were

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indications that this tolerance was not stable. If skin was grafted at the time of, or immediately after, the termination of the parabiosis, it showed prolonged survival but eventually began to be rejected. Shaw et al., (1974) repeated and confirmed these observations and added the information that the in vitro MLR test between parabiont spleen cells and normal C3H/HeJ spleen cells remained positive, despite the state of in vivo unresponsiveness. This suggested that the state of unresponsiveness in parabiont mice of this strain combination was not similar to that seen with neonatally tolerant mice. The suggestion was also advanced by Shaw et al., (1974) that the F1 parabiont spleen was colonized by parental cells and that these cells remained immunocompetant; this extended McBride and Simonsen's (1965) observation of spleen cell takeover to include the retention of function.

Thus DBA/2J-plus-(DBA/2J x C3H/HeJ)F1 parabionts presented two unexplained characteristics: anti-F1 immuno recognition was retained although no obvious harm to the F1 was done by it and, parental cells were hypothesized to be able to actually replace the immune system, at least as judged by the behavior of the spleen in vitro, of the F1 partner. These two facets, the unresponsiveness, and the takeover, were selected for detailed study. The intention of this project was to define more carefully the range of retained immune reactivities in the parabionts, whether both partners retained them, whether subregions within the H-2

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complex of the "target" mouse (the F1) were critical, and, as the project developed, whether the unresponsiveness could be accounted for on the basis of a suppressor cell, or akin to enhancement, whether an antibody was important. A second and parallel investigation focused on the takeover process: did it extend to the circulating red cell system as well as the lymphoid system, could it be shown directly as well as functionally, and, in addition, what relationship, if any, did the presence of parental immunocompetence against the F1 partner have in triggering the takeover process. It will be the assertion at the end that immunological enhancement may play the crucial role in parabiont tolerance in this strain combination.

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MATERIALS AND METHODS

Mice BALB/cCR (H-2d), DBA/2J (H-2d), C3H/HeJ (H-2k), CBA/J (H-2k), CBA/CaJ (derived from CBA/H, H-2k), C3H.NBSn (H-2p), and C3H.SWSn (H-2b) were obtained from The Jackson Laboratory, Bar Harbor, Maine, and maintained as pedigree lines in our mouse colony (Laboratory Animal Breeding Unit, Ellerslie Experimental Station, Ellerslie, Alberta) except for C3H.NBSn which was maintained in the Dentistry-Pharmacy Animal Breeding Unit at the University of Alberta. Breeding stock of C3H.A/Ha (H-2a) and C3H.OH/Ha ($H\text{-}2^{o2}$) were obtained from Dr. T.S. Hauschka (whom we gratefully acknowledge) at the Roswell Park Memorial Institute, Buffalo, New York. The C3H.A and C3H.OH strains have been maintained since by the Dentistry-Pharmacy Animal Breeding Unit. H-2, GPI-1, and Mls genotypes are given in Table 1. F1 hybrids were bred as needed.

Antisera An antiserum to C3H/HeJ (H-2k) was raised in (DBA/2J x C3H.SWSn)F1 (H-2d x H-2b) mice with a series of weekly intraperitoneal injections of C3H/HeJ spleen cells. This antiserum, after absorption with (DBA/2J x C3H.SWSn)F1 packed spleen cells, had a cytotoxic titer against C3H/HeJ of 1:724. Selected, nontoxic rabbit sera were used as sources of complement.

CBA/J anti-L1210 (a leukemia syngeneic to DBA/2J) was

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TABLE 1

H-2, GPI-1, AND MLS GENOTYPES OF MICE USED IN THIS STUDY

	H-2									GPI-1	Mls
	K	I-A	I-B	I-J	I-E	I-C	S	G	D		
DBA/2J	d	d	d	d	d	d	d	d	d	a	1
C3H/HeJ	k	k	k	k	k	k	k	k	k	b	3
(DBA/2J x C3H/HeJ)F1	d/k	d/k	d/k	d/k	d/k	d/k	d/k	d/k	d/k	a/b	1/3
C3H.NB	p	p	p	p	p	p	p	p	p	b	3
C3H.A	k	k	k	k	k	d	d	d	d	b	3
C3H.OH	d	d	d	d	d	d	d	d	k	b	3
C3H.SW	b	b	b	b	b	b	b	b	b	b	3
CBA/J	k	k	k	k	k	k	k	k	k	b	4
CBA/CaJ	k	k	k	k	k	k	k	k	k	b	2
BALB/cCR	d	d	d	d	d	d	d	d	d	a	2

(References: Freed, et. al., 1976; Klein, 1975).

used as an anti-H-2d antiserum. Its cytotoxic titer was 1:1000. Its reactivity to a panel of C3H congenic mice indicated that the antiserum was directed solely against products of the H-2 complex (unpublished observations). Both antisera were used at a dilution of 1:50.

Hybridoma-Derived Monoclonal Anti-H-2K(k) The 11-4.1 hybridoma cell line, originating in the laboratory of Dr. L.A. Herzenberg (Stanford University School of Medicine, Stanford, California), was obtained from The Salk Cell Distribution Center, La Jolla, California. It secretes an IgG2a antibody, reactive with H-2K(k) but not H-2K(d)

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antigen bearing cells (Oi, et al., 1978). Further characterization is described in Wegmann, et. al., (1979). Raw ascites fluid from BALB/cCR mice in which this hybridoma was growing, was used directly; it was the kind gift of Dr. T.R. Mosmann.

Tumors Two tumors were used for some of the experiments described. The RI leukemia (H-2k, and see Olivotto and Bomford, 1974) and the L1210 leukemia (H-2d) were maintained, in ascites form, by serial weekly passage in C3H/HeJ and (BALB/cCR x DBA/2J)F1 mice respectively. The RI tumor originated in a CBA/H mouse but was passaged more than 25 times through C3H/HeJ mice before use. Weekly passage of 1-5 x 10^6 tumor cells was done in Phosphate Buffered Saline (PBS).

Parabiosis Parabiosis (Figure 1) was performed as previously described (Bunster and Meyer 1933, Shaw et. al., 1974) except that tail skin grafting was not routinely performed. At the time of parabiosis, all animals were age, sex, and size matched as closely as possible with particular emphasis given to size matching; the mice were 10 weeks of age or older when joined. Both male and female pairs were used. Only parabionts that had survived for more than 100 days were used for most of the experiments described. In most pairs studies, the complete takeover of the F1 hybrid partner's red blood cell system by parental cells was confirmed by starch gel electrophoretic analyis of red blood cell lysates for isozymes of glucose phosphate isomerase

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Figure 1.

DBA/2J (H-2d, on the right in this photograph) plus (DBA/2J x C3H/HeJ)F1 (H-2d x H-2k; here, on the left) parabiosed mice. These mice have been in parabiosis for more than 100 days.

(E.C. 5.3.1.9) as described below. This takeover is characteristic of all successful parabionts of our most studied strain combination, DBA/2J plus (DBA/2J x C3H/HeJ)F1.

Hematocrit Determinations

Under light Penthrane (methoxy fluorane, N.F., Abbott Laboratories, Montreal, Quebec, Canada) anaesthesia, mice were bled from the retro-orbital sinus into micro hematocrit tubes (Yankee #1020, Clay-Adams, New Jersey), which were plugged at one end with Critoseal (Clay-Adams, New Jersey) and spun in a hematocrit centrifuge for 10 minutes (model MB, International Equipment Co., Needham, Mass.). The proportion of red blood cells was then determined by direct measurement.

Red Blood Cell Lysates and Starch Gel Electrophoresis for Isozymes of Glucose Phosphate Isomerase

DBA/2J differs at the GPI-1 locus from all of our strains of C3H as well as both strains of CBA (DeLorenzo and Ruddle, 1969 and Table 1). The GPI-1a allele, carried by DBA/2J, determines a form of the enzyme glucose phosphate isomerase (E.C. 5.3.1.9) which migrates more slowly towards the cathode on starch gel electrophoresis at pH 6.2. The C3H and CBA strains carry the GPI-1b allele which determines a more rapidly migrating form. F1 hybrids (GPI-1a/GPI-1b heterozygotes) display an intermediate band along with the two parental bands. In artificial DBA:F1 mixtures, we can

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detect the presence of F1 enzyme if it is not less than 10% of the mixture.

For the preparation of RBC lysates, blood was obtained from the retro-orbital sinus, washed twice in PBS, and the packed red cells lysed with distilled water (1:1 v/v). This lysate was centrifuged in a Beckman 152 microfuge for 5 minutes and the supernatant aliquoted and frozen at -80°C until analyzed.

The conditions of starch gel electrophoresis were essentially those of Chapman, et. al., (1971) with slight modifications. Lysates were run for 4 hours, in the cold, at 130 - 150 volts DC across plastic tanks filled with tris-citric acid running buffer (2.4% w/v Tris base, 2.6% w/v citric acid, adjusted to pH 6.2 with 12N HCl). The glucose phosphate isomerase patterns were then developed with the specific activity stain (Chapman et. al., 1971).

Bone Marrow Chimeras Mouse bone marrow chimeras were produced essentially by the technique of Sprent, et. al., (1975). We observed high survival of the chimeras if the bone marrow inoculum was depleted of debris and dead cells, following the anti-Thy-1 (the kind gift of Dr. Chiaki Shiozawa) and complement treatment, by centrifugation on a Ficoll-Hypaque gradient before injection into the lethally irradiated recipients. The Ficoll-Hypaque gradient centrifugation was performed exactly as described in Pope, et. al., (1976) for spleen cells. All chimeras were made by

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injecting 10^7 anti-Thy-1 treated DBA/2J bone marrow cells into (DBA/2J x C3H/HeJ)F1 hybrids. Prior to bone marrow reconstitution, the recipients were irradiated with 850 Rads of whole body irradiation delivered at the rate of 100 Rads per minute, from a ^{137}Cesium source (Gamma Cell 40, Atomic Energy of Canada, Ltd.)

Fetal Liver Chimeras These chimeras were made by injecting 10^7 10-13 day old DBA/2J fetal liver cells in Liebovitz medium (G1BCO, L-15) into 900 Rad irradiated (DBA/2J x C3H/HeJ)F1 (H-2d x H-2k) or (DBA/2J x C3H.NBSn)F1 (H-2d x H-2p) mice, approximately 16 months before use. These mice share the immunological characteristics of bone marrow chimera mice and are easier to make.

Injection Chimeras These chimeras were made by injecting about 500 x 10^6 parental strain spleen cells, suspended in Liebovitz medium at 250 x 10^6 cells per ml. into adult H-2 incompatible F1 hybrids. Most commonly, DBA/2J cells were injected into (DBA/2J x C3H/HeJ)F1 mice but occasionally other parent-F1 combinations were used. The F1 hybrids were untreated adults generally 3 months of age or older. 0.1 ml heparin (Hepalean, Heparin sodium, Harris Laboratories, Toronto, Ontario DIN 338575, 1000 units per ml diluted 1:10 in saline) was injected i.p. immediately before i.v. injection of spleen cells.

Cell Mediated Cytotoxicity These assays were done according to the method of Brunner et. al., (1968) with several modifications. Briefly, for direct assay of splenic cell

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cytotoxicity, spleens from both parent and F1 partners, in parabiosis for more than 100 days, were removed into Liebovitz medium (GIBCO, L-15) supplemented with 10% heat inactivated Fetal Calf Serum (FCS, GIBCO). Single cell suspensions were prepared by gently pressing the spleens through metal screens; the debris and cell clumps were removed by allowing them to settle for 7 minutes. The remaining cells were then washed once or twice in Liebovitz medium by centrifugation at 1100 rpm for 7.5 minutes. After a final centrifugation step, the cells were resuspended at a concentration of 2 x 10^6 cells per ml in Mishell-Dutton medium (Mishell and Dutton, 1967).

Eagles' Minimal Essential Medium (MEM) was made and supplemented with 10% heat inactivated fetal calf serum, sodium pyruvate (10 mls per liter of a 100 mM stock, Flow Laboratories, cat. no. 16-820-49), Pen-Strep (1 ml of a 10,000 unit per ml stock, GIBCO, #514) and sodium bicarbonate (0.35%). Before use, this medium was neutralized to about pH 7.4 with CO2 gas and sterilized by filtration through a Millipore apparatus.

The target cells were spleen lymphocytes made up in MEM with supplements, at a concentration of 15 x 10^6 cells per ml, stimulated with phytohemagglutinin (PHA, Wellcome Research Laboratories, Beckenham, England) at a final dilution of 1:50. One ml of these cells was placed in the inner compartment of a Marbrook apparatus (Marbrook, 1967,

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as modified by Diener and Armstrong, 1967) with 50 mls of supplemental MEM as a reservoir of nutrients. This was incubated at 37°C in a 10% CO2 incubator at 95% humidity for two days. Generally four flasks were set up for each set of targets.

After PHA stimulation, the target cells were harvested and washed once in MEM. 5 x 10^6 cells were concentrated into 0.5 ml of MEM. To this was added 100 microcuries of $Na^{51}CrO4$ (New England Nuclear); this was incubated for 50 minutes, washed 3 times in MEM and adjusted to 5 x 10^5 cells per ml. 0.1 ml of this suspension was added to a 12 x 75 mm test tube (Canlab, T1290-3) containing 2.5 mls of cells (5 x 10^6 cells) whose cytotoxicity was being assessed. This suspension was divided into 5 tubes of 0.5 mls each, and incubated at 37°C for 6 hours. Then 1 ml of an 0.2% suspension of sheep erythrocytes in Mishell-Dutton medium was added to each tube. After brief agitation, the tubes were centrifuged at 1100 rpm for 7 minutes, and 1 ml of the supernatant from each tube transferred to a fresh test tube, which was then counted for gamma irradation in a Packard Autogamma Spectrometer Model 3002 (Packard Instruments of Canada, Montreal, Quebec).

Both spontaneous release (^{51}Cr labeled target cells with no added responder cells) and freeze-thaw release (^{51}Cr labeled target cells frozen and thawed three times) were included in every experiment.

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For assaying the in vitro development of cytotoxicity, spleen cells were prepared under sterile conditions in supplemented MEM as before, but at a concentration of 15 x 10^6 cells per ml. Stimulator spleen cells, usually from a donor mouse given 950 Rads of irradiation, were prepared exactly as described for responder lymphocytes, in supplemented MEM at a final cell concentration of 20 x 10^6 per ml. 1 ml of responder cell suspension plus 0.1 ml of stimulator cell suspension was added in the inner compartment of modified Marbrook flasks with about 50 mls of supplemented MEM in the reservoir. Usually each type of culture consisted of four such flasks. These were incubated at 37°C for 5 days, harvested by pooling flasks of each group, and assayed for ^{51} Cr release as described.

The percent specific release was calculated from the formula:

$$\frac{X \text{ (mean experimental release)} - SR \text{ (spontaneous release)}}{FT \text{ (mean freeze thaw release)} - SR \text{ (spontaneous release)}}$$

Dye Exclusion Cytotoxicity Tests These were done according to the method of Boyse, et. al., (1964) as modified by Carlson and Terres (1976) except that Liebovitz medium was used instead of medium 199. Most often, spleen cell targets were used, harvested as described (Boyse, et. al., 1964) including a 5 minute incubation at 37°C in 0.83% NH4Cl to lyse contaminating erythrocytes (Boyle, 1968). For some tests, tumor cells were used for targets; for this purpose,

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1-5 x 10^7 tumor cells (either RI or L1210) were injected i.p. (into either C3H/HeJ or (BALB/cCR x DBA/2J)F1 mice respectively). This resulted, two days later, in a plentiful supply of tumor cell targets without significant host red blood cell contamination. For the cytotoxic titration, tumor cells were harvested in Liebovitz medium containing 0.1% gelatin.

Local Graft-verus-Host Assays

The popliteal lymph node enlargement assay for local GVH reactivity was used (Twist and Barnes, 1973; Binz, 1975). The parabiont donors of cells for these experiments ranged from 113 to 254 days duration in parabiosis. No influence of the duration of parabiosis on GVH reactivity was observed. The right rear footpads of appropriate F1 hybrids received injections of 1.5 x 10^7 parabiont spleen cells (or, for positive controls, normal DBA/2J spleen cells), and the left rear footpads received an equal number of appropriate normal F1 spleen cells. The spleen cells were administered in 0.05 ml Liebovitz medium to each footpad. Seven days later, both footpads were injected with 0.05 ml of 5% Trypan Blue dye in saline. The popliteal lymph nodes were removed and weighed, and a weight ratio (right/left) obtained.

Adoptive Transfers DBA/2J mice (5/group) of both sexes were irradiated with 850 R at the rate of 100 R per minute and reconstituted one day after irradiation with normal DBA/2J spleen cells, or parabiont spleen cells pooled from the

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parent and F1 partners. The standard reconstitution was 10^7 cells in 0.5 cc of Liebovitz medium, injected i.v. In those groups to be stimulated with C3H/HeJ alloantigens, 10^7 irradiated (1200 R) C3H/HeJ spleen cells were injected i.p. at the time of restoration with DBA/2J or parabiont cells. Five days later, the ^{131}I-labeled tumor cells were injected i.p. and monitoring of the response was begun.

Tumor Labeling and In Vitro Monitoring of Alloimmunity The origin and maintenance of the RI tumor (derived from CBA/H (H-2k), but maintained in C3H/HeJ for more than 25 passages) have been described (Olivotto and Bomford, 1974). It was used here to monitor immunity to H-2k transplantation antigens. The method has been previously described (Hofer, et. al., 1969; Forman et. al., 1972; Terres, et. al., 1960). Briefly, 10^6 RI tumor cells were injected i.p. into a C3H/HeJ mouse 6 days prior to labeling. Then the RI tumor was labeled in vivo by i.p. injection of 70-100uCi of ^{131}I-iododeoxyuridine (^{131}I-IUdR) 2-4 hours before harvest. After harvesting and washing, in Earles' Balanced Salt Solution (EBSS, Cat. no. K-11, GIBCO, New York), 10^6 cells containing approximately 50,000 cpm of ^{131}I, were injected i.p. in a volume of 0.2 cc of EBSS into the experimental mice. The mice were restrained in tubes and counted daily in a Beckman Gamma 300 NaI crystal scintillation counter with the elevator in the low position. All mice injected with labeled tumor cells were maintained on drinking water with 0.1% NaI (w:v) to minimize thyroid uptake of radioactive iodine

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(Terres, et. al., 1960). Individual mice were gamma-counted after injection of the labeled tumor cells to determine the initial amount of injected radioactivity (100% for day 0 in the Figures) and daily thereafter.

Since intact parabiont pairs cannot be whole-body counted in the Gamma 300, they were injected i.p. with 10^6 ^{131}I-tumor cells (10^6 cells into each partner) and set aside for three days. On the third day, the parabionts were sacrificed, separated, and each partner counted. The percent retention of ^{131}I was calculated using the average day 0 counts of the control mice according to the formula:

$$\frac{\text{[parabiont cpm] x [isotope decay correction]}}{\text{mean of day 0 cpm of control mice}} \times 100$$

When tumor clearance data are given as the percent of tumor killed, these values are calculated according to the formula:

$$(c - x)/c$$

where c is the percent of ^{131}I-RI retained in the control nonimmune group and x is the percent retained in the experimental group or individual.

The validity of ths method for assessing in vivo immunity has been shown (Carlson and Terres, 1976; Hofer, et. al., 1969; Forman, et. al., 1972; Terres, et. al., 1960).

Skin Grafting: Flank skin grafts were done as described by Baldwin et. al., (1973).

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RESULTS

I. Hematologic Consequences of Parabiosis

1. Red Blood Cell Takeover

Red blood cell lysates have been analyzed from many DBA/2J and (DBA/2J x C3H/HeJ)F1 parabionts, together for more than 100 days. These lysates were examined by starch gel electrophoresis. Using artificial mixtures, the sensitivity of the starch gel technique was such that the C3H component of the F1 GPI could easily be detected in a lysate mix that was 90% DBA/2J and 10% F1. At the 10% level of resolution, none of the lysates from DBA/2J parabionts had any detectable F1 GPI. Figure 2 shows the banding pattern obtained with lysates from three representative F1 parabionts, a normal DBA/2J, a normal F1, and an artificial 1:1 DBA/2J and F1 mixture. The three F1 parabionts depicted here showed only DBA/2J-type GPI activity, and the same was found to be true for all of 22 F1 parabionts tested.

In general, this takeover process required between six and nine weeks to complete. In two pairs in which the DBA/2J partners died at 5 and 8 weeks, the F1 partners were successfully separated and maintained until GPI patterns were analyzed 11 and 5 weeks later respectively. In both separated F1 mice, complete takeover had occurred. In one other pair, separated at 13 days after parabiosis, the F1 partner 90 days later showed no takeover at all. Thus a

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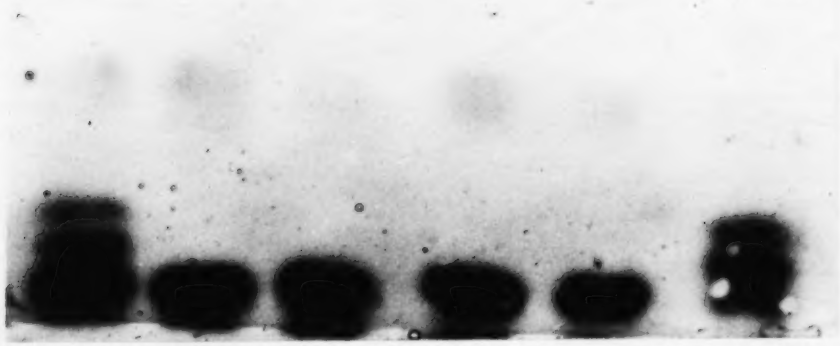

1:1 Artificial Mix DBA:F1	F1*#1	F1*#2	F1*#3	Normal DBA/2	Normal (DBA/2 x C3H/HeJ)F1
	----Parabiosed F1s---				

Figure 2.

Starch gel electrophoresis of glucose phosphate isomerase (GPI) from red blood cell lysates from adult (DBA/2J x C3H/HeJ)F1 hybrid mice in parabiosis with adult DBA/2J mice. The characteristic 1:2:1 staining pattern of normal F1 GPI is seen on the right. Normal DBA/2J migrates in a single slow moving band as seen immediately next to the normal F1 pattern. An artificial 1:1 mix of DBA/2J with F1 migrates as seen on the far left. The intermediate three bands show the migration patterns of GPI from lysates of three representative F1 parabionts. The identity of these migration patterns with that of a normal DBA/2J is evident.

minimum period of parabiosis may have been required to establish the takeover reaction although the number of mice upon which this is based is small. As a consequence of this period of time in parabiotic union, DBA/2J red blood cells, or their precursors, were capable of crossing into the F1 partner and establishing themselves, and simultaneously F1 red blood cell production was suppressed. All this occurred without perceptibly affecting the health of either partner (Drell, et al., 1975).

2. The Takeover Reaction Includes Spleen Lymphocytes as Well as RBC

To determine the extent of parental lymphocyte takeover, dye exclusion cytotoxicity tests on parabiont spleens, using the (H-2d x H-2b)F1 anti-H-2K antiserum, were performed. As shown in Table 2, no F1 cells bearing H-2K alloantigens could be found in the spleen of either partner within the limits of detection of this technique. Control experiments with artificial spleen cell mixtures indicated that F1 hybrid cells could be detected if present in as low a proportion as 10%. Table 2 also shows that, using the H-2K anti-H-2d antiserum, virtually all lymphocytes present in the spleen of either parabiont partner were H-2d in phenotype and therefore derived from the parental partner. Table 3 shows that the same conclusions hold true for parabiont bone marrow lymphocytes. An analysis of variance test indicated that after treatment with anti-H-2K and complement, no significant differences in lysis were present

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Table 2. Dye exclusion cytotoxicity tests on spleen lymphocytes from normal and parabiont DBA/2J and (DBA/2J x C3H/HeJ)F1 mice

	Percent lysis (SD)			
	P462;225 days*	P494;216 days	P501;240 days	P687;155 days
Anti-H-2d				
DBA normal	92.4(3.1)	94.2(1.3)	>95	93.0(2.8)
F1 normal	89.8(3.3)	95.6(2.2)	>95	79.0(7.8)
DBA parabiont	80.0(3.5)	-ND -	>95	91.2(2.2)
F1 parabiont	87.7(2.3)	85.6(7.0)	>95	93.0(2.7)
Anti-H-2k				
DBA normal	13.8(3.0)	15.6(5.2)	13.4(3.0)	7.0(1.4)
F1 normal	82.2(3.6)	92.4(2.8)	>95	80.2(7.0)
DBA parabiont	16.6(4.5)	-ND -	14.0(3.1)	8.8(1.5)
F1 parabiont	9.4(2.7)	17.4(1.7)	18.4(4.7)	8.4(2.9)

* Duration of parabioses. (P462 is the code for a single pair of mice in parabiosis)
Statistical analysis (analysis of variance) was performed by Mr. Brian Pinchbeck of the University of Alberta Computing Services. With anti-H-2d antisera, no significant differences in lysis of any of the four cell types were seen. With anti-H-2k antisera, no significant differences in lysis of DBA normal, DBA parabiont and F1 parabiont spleen cells were seen. F1 normal spleen cells showed lysis which was highly significant with respect to the other three cell types ($P < 0.002$). Lysis of target cells by complement alone never exceeded 15% and was usually around 10%.
ND = not done.

between DBA/2J normal, DBA/2J parabiont, or F1 parabiont spleen cells and that all three cell types showed lysis which was highly significantly different from the lysis of F1 normal spleen cells ($P < 0.002$). These experiments are a direct indication that hybrid - derived lymphocytes are not present in detectable numbers in the spleen of either partner in parabionts of this strain combination.

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Table 3. Dye exclusion cytotoxicity tests on bone marrow cells from normal and parabiont DBA/2J and (DBA/2J x C3H/HeJ)F1 mice

	Percent lysis (SD)			
	P439;187 days[1]	P446;190 days	P501;240 days	P701;264 days
Anti-H-2d				
DBA normal	-ND-[5]	51.2(2.6)	95.2(6.7)	42.0(17)
F1 normal	-ND-	55.5(11.0)	85.8(12.0)	29.8(18.9)
DBA parabiont	58.0(8.2)	55.0(9.1)	-ND-	22.8(3.0)[4]
F1 parabiont	58.2(5.0)	54.0(7.0)	88.2(5.4)	40.6(15.6)
Complement control	26.0	23.0(3.4)	16.0(1.7)	<5
Anti-H-2k				
DBA normal	27.5(8.7)	17.2(4.,0)	16.8(2.5)	8.4(3.2)[3]
F1 normal	59.0(12.0)[2]	53.5(5.7)[2]	83.2(4.5)[2]	36.6(3.7)[2]
DBA parabiont	29.8(3.1)	27.2(5.4)	-ND-	6.0(2.2)
F1 parabiont	31.0	23.0(4.2)	16.4(4.2)	8.8(3.4)[3]
Complement control	25.2(11.8)	23.5(9.9)	14.7(4.7)	<5

1. Duration of parabiosis.
2. Value is significantly ($P < .05$) different from complement control.
3. Percent lysis values are significantly ($P < .05$) different from complement control. All other lysis values for DBA normal, DBA parabiont, or F1 parabiont bone marrow cells, incubated with anti H-2k and complement, do not differ significantly from the complement control.
4. Percent lysis value for DBA parabiont bone marrow cells is significantly different ($P < .025$) from the lysis of F1 parabiont bone marrow cells. In all other direct comparisons between DBA and F1 parabiont bone marrow cells, treated with either antiserum, the lysis did not significantly differ.
5. ND: not done.

3. The Takeover Reaction Requires a Nontolerant Parental Partner

It was of considerable interest to know whether, in the complete absence of immunological recognition of the hybrid

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partner by the parent, a takeover would occur. One possible explanation of the takeover reaction was that hemopoietic stem cells from the DBA/2J partner enjoy an intrinsic competitive advantage over F1 stem cells in the F1 hybrid environment. We therefore made radiation chimeras by injecting anti-Thy-1 and complement treated DBA/2J bone marrow cells into lethally irradiated (850-900 R) (DBA/2J x C3H/HeJ)F1 hybrids, following the method of Sprent, et al. (1975). (It is a pleasure to acknowledge the valuable assistance of Dr. Sue Rubinstein in construction of these chimeras). Eight weeks later, all the chimeras were tested for RBC chimerism by the GPI isozyme assay and found to be almost entirely taken over by the donor inoculum. Only traces of hybrid isozyme were found, which were accountable for on the basis of the known 23 day half-life of mouse red cells (Russell and Bernstein, 1966). Twenty of these chimeric F1 hybrids were then parabiosed to normal syngeneic (DBA/2J x C3H/HeJ)F1 hybrids. Fifteen pairs were tested for RBC chimerism at 12 weeks after parabiosis, a time when, in conventional parabionts, the takeover reaction is complete (vide supra). There was no takeover of either partner by cells from the other in all of the 15 pairs. The GPI patterns were indistinguishable from artificial 1:1 mixtures of DBA/2J and F1 hybrid isozymes. This is shown in Figure 3a and b.

The reactivity of the originally chimeric F1 partners towards normal F1 cells bearing C3H/HeJ antigens was tested

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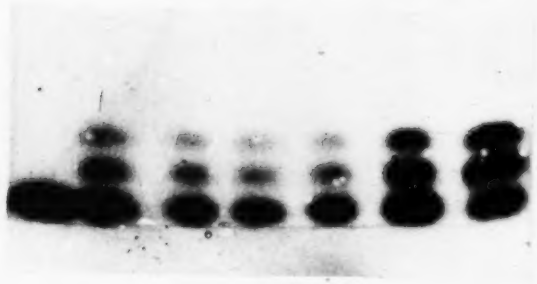

DBA nF1 #1 | F1* nF1 #1 | F1* nF1 #2 | F1* #2 | 1:1 Mix DBA: F1 | Normal F1

Figure 3a.

Starch gel electrophoresis of glucose phosphate isomerase from RBC lysates of four bone marrow chimera parabionts. 'F1*' is the DBA/2J --> (DBA/2J x C3H/HeJ)F1 bone marrow chimera partner. 'nF1' is the untreated (DBA/2J x C3H/HeJ)F1 parabiosed to the bone marrow chimera partner. The 1:1 mix is an artificial mixture of normal DBA/2J and normal F1 (unparabiosed) lysates. RBC lysates were made 12 weeks after parabiosis. These patterns are representative of 15 such pairs tested.

Figure 3b.

See legend to Figure 3a.

Normal F1 | 1:1 Mix DBA: F1 | F1* #3 | nF1 #3 | F1* #4 | nF1 #4 | DBA

in the local graft-versus-host (GVH) assay (popliteal lymph node enlargement), using normal (DBA/2J x C3H/HeJ)F1 hybrids. Four pairs were tested and, in two of these, the chimeric F1 partner gave slight but statistically significant lymph node enlargement (Figure 4a). This might reflect a low level of reactivity in some bone marrow chimeras, related to the MLR seen by Sprent, et. al., (1975) in their "one-way" parent to F1 chimeras. This slight reactivity is in contrast to the normal GVH reactivity seen against (DBA/2J x C3H.SWSn)F1 (H-2d x H-2b) recipients (Figure 4b) and the significant reactivity seen in conventional DBA/2J plus F1 parabionts (vide infra).

These experiments indicate that for a takeover of the hybrid partner to occur, the cells of the DBA/2J partner must be able to immunologically recognize C3H/HeJ antigens. When these cells are made unresponsive towards C3H/HeJ, they cannot mediate the takeover reaction (Drell and Wegmann, 1979).

4. Hematocrit Profiles

Figure 5 shows the profile of hematocrits, as a function of time after parabiosis, of the DBA/2J with (DBA/2J x C3H/HeJ)F1 combination. The profile of the hematocrits from only those pairs that survived to 100 days, while not shown, closely resembles that shown in Figure 5. For roughly the first 4 weeks, the parental partners show the classical pattern of polycythemia and the F1s show

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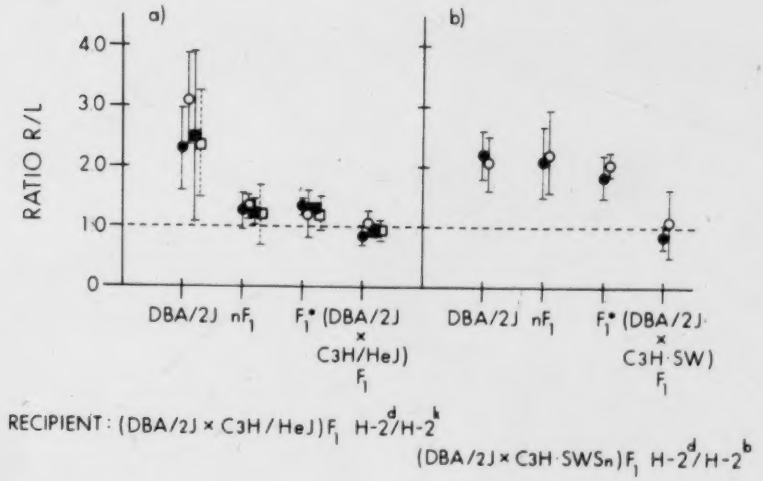

Figure 4.

Local graft-versus-host reactivity of spleen cells from bone marrow chimera (DBA/2J marrow into (DBA/2J x C3H/HeJ)F1: Normal (DBA/2J x C3H/HeJ)F1) parabionts. 1.5 x 10^7 spleen cells in Liebovitz medium were injected into each footpad. The right rear footpad received experimental cells (indicated on abcissa), while the left rear footpad always received syngeneic F1 spleen cells as a control. Seven days later, the popliteal lymph nodes were removed and weighed. The ordinate indicates the ratio of the weight of the draining lymph node from the footpad injected with experimental cells to the weight of the node from the footpad injected with syngeneic F1 cells. Data are means $\pm$ SD of at least 4 mice per point, with the sole exception of the chimeric F1 partner (F1*) into (DBA/2J x C3H/HeJ)F1 recipients in Exp. 3. A ratio of 1.0 indicates no lymph node enlargement. a) shows the retention of only slight to no reactivity, in both partners, (nF1 is the normal F1 partner, F1* is the marrow chimera partner) towards the alloantigens to which tolerance was induced. b) shows the retention of normal levels of third party GVH reactivity. In both panels, DBA/2J represents the positive control, i.e., spleen cells from normal DBA/2J mice. The syngeneic F1 is the analagous negative control. The four different symbols represent four separate experiments.

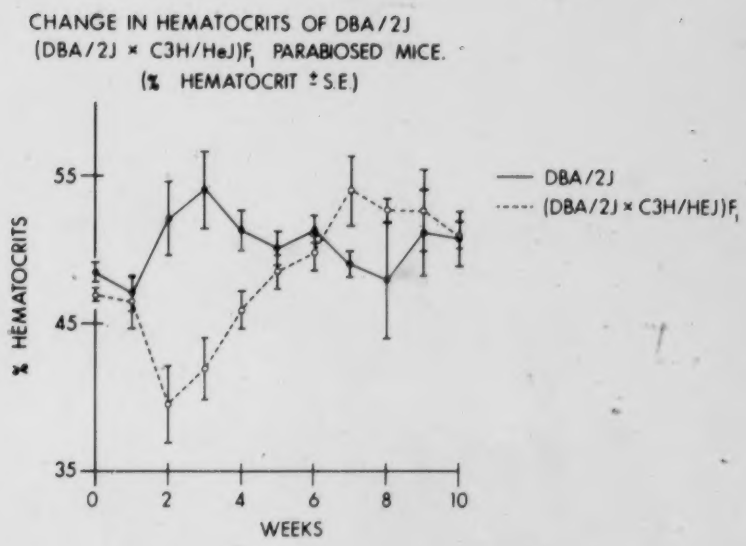

Figure 5.

Change in hematocrits (% ± S.E.M.) with time after parabiosis, of DBA/2J (H-2d) with (DBA/2J x C3H/HeJ)F1 (H-2d x H-2k) parabiosed mice. Values for unparabiosed controls are given on the ordinate.

anemia. Then, however, in those parabionts surviving to 5 weeks and more, the hematocrit curves begin to reach an equilibrium and are subsequently stable albeit at a level slightly above normal (Wegmann and Drell, 1975). Each point in Figure 5 is based on at least 9 parabiont partners.

Thus, in DBA/2J plus (DBA/2J x C3H/HeJ)F1 parabionts, a takeover of the F1 erythropoietic system occurs after parabiosis and remains stable with time. Others have shown variable degrees of "takeover" (i.e. presence of parental partner cells in the F1 partner) either using T6 chromosomally marked cells (Nakic, et al., 1966, Nisbet 1971) or direct dye exclusion cytotoxic analysis of cells in the F1 spleen (McBride and Simonsen, 1965). In this system, we have demonstrated a complete (to the level of sensitivity of our assays) takeover of the spleen, the bone marrow, and circulating red cells of the F1 by parent-derived cells and also shown that immunocompetence is required for the DBA/2J partner to effect this takeover. The anemia-polycythemia syndrome so frequently observed in H-2 incompatible parent-F1 parabiosis (Eichwald, et. al., 1960, 1961; Nisbet, 1973) is present in this strain combination also but resolves with time. This may correspond temporally with the elimination of sufficient F1 lymphocytes and RBC to remove further stimulation of the DBA/2J response. The relationship, if any, of the anemia-polycythemia to the takeover reaction is not clear.

II. Genetic Influences on Parabiosis

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1. Long Term Parabiont Survival and Parental Takeover Vary with the Target Haplotype Presented in the F1 Hybrid Partner

a) H-2

That genetic differences between parabiont partners are important was first demonstrated by Finnerty and Panos (1951). The importance of H-2 was shown by Eichwald, et. al., (1959, 1963). That the DBA/2J plus (DBA/2J x C3H/HeJ)F1 strain combination was remarkable was discovered by Rubin (1959) who found a 40% survival rate (29/71).

As can be seen in Figure 6, approximately half of the DBA/2J with (DBA/2J x C3H/HeJ)F1 parabionts in this series survive to 100 days and beyond. Survival to 100 days after parabiosis is the invariant criterion for success. This percentage is entirely consistent with Rubin's (1959) and based on many more pairs (136 in Figure 6, 268 pairs in subsequent Figures).

Figure 6 also shows the significantly higher percentage of survival at 100 days of the DBA/2J with (DBA/2J x C3H.NBSn)F1 parabionts (about 69%, $p < 0.02$). Since C3H.NBSn differs from C3H/HeJ mainly at the H-2 complex, this differential survival can most likely be ascribed to the influence of the H-2p haplotype. Since C3H.SWSn also differs from C3H/HeJ mainly at H-2, the much lower survival percentage of DBA/2J with (DBA/2J x C3H.SWSn)F1 parabionts (16%, $p < 0.02$) is again most likely a result of the presence

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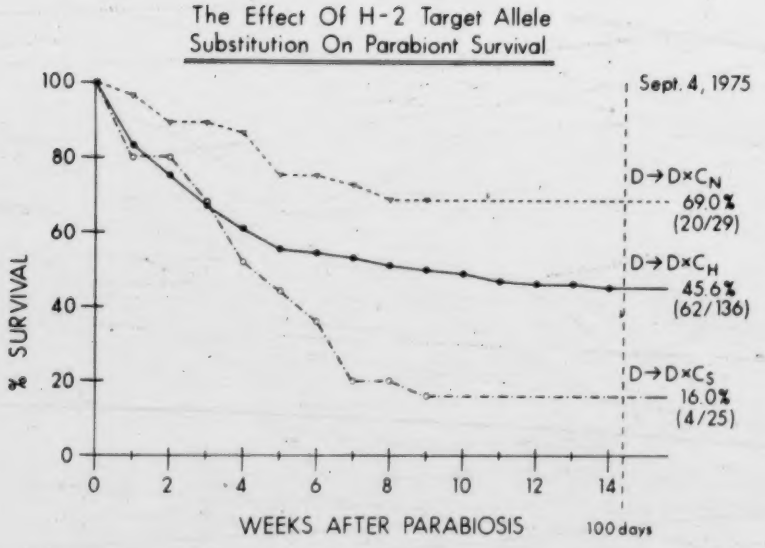

Figure 6.

Survival curves (number surviving to a given week, divided by total parabionts of the relevant strain combination, x 100) for three different combinations of histoincompatible parent with F1 parabiosis, as a function of time after parabiosis. "D --> D x CN" = DBA/2J (H-2d) plus (DBA/2J x C3H.NBSn)F1 (H-2d x H-2p). "D --> D x CH" = DBA/2J plus (DBA/2J x C3H/HeJ)F1 (H-2d x H-2k). "D --> D x CS" = DBA/2J plus (DBA/2J x C3H.SWSn)F1 (H-2d x H-2b).

of the H-2b haplotype in this combination. Thus, the H-2 composition of the F1 hybrid parabiont can significantly alter its survival in parabiosis with the DBA/2J parental strain (Drell and Wegmann, 1976).

A caution must be noted here about congenic mice. Conclusions about the role of marker genes by which congenic mice differ from the reference strain are necessarily limited by uncertainty about the exact location of the crossover points outside the detectable marker loci. In the production of many H-2 congenics, a considerable amount of "passenger" genetic information on chromosome 17 can accompany the H-2 marker complex (Bailey, 1970; Klein, 1975) and it is possible that linked "silent" genes might influence the behavior for which congenic mice differ from the reference line. If this were so, it would be incorrect to ascribe the observed differences in parabiont survival, etc., to the H-2 complex. The strictly valid conclusion is that the observed differences are due to genetic information by which the congenic and reference strains differ.

The pattern of hematocrits for DBA/2J with (DBA/2J x C3H/HeJ)F1 parabionts is shown in Figure 5, showing the distinctive anemia - polycythemia during the early weeks. The pattern in DBA/2J with (DBA/2J x C3H.NBSn)F1 parabionts is markedly different (Figure 7). Here, there is no classical profile of parental polycythemia and F1 anemia. Except for a small dip at 2 weeks, both partners exhibit

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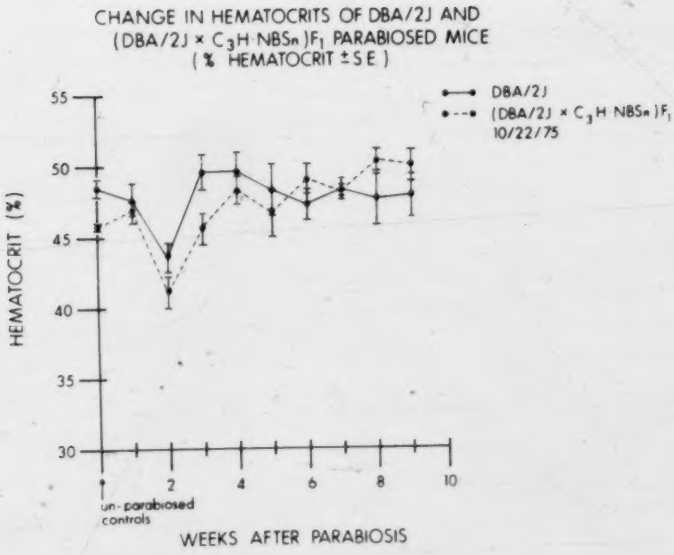

Figure 7.

Change in hematocrits (% ± S.E.M.) with time after parabiosis of DBA/2J (H-2d) with (DBA/2J x C3H.NBSn)F1 (H-2d x H-2p) parabiosed mice. Values for unparabiosed controls are given on the ordinate.

stable hematocrits through to 9 weeks. Not enough DBA/2J with (DBA/2J x C3H.SWSn)F1 parabionts survive to test the hematocrit profile of this strain combination.

As previously noted above, DBA/2J with (DBA/2J x C3H/HeJ)F1 parabiosis routinely leads to an apparently complete parental takeover of the F1 hemopoietic system as determined by starch gel electrophoresis of red blood cell lysates. In DBA/2J with (DBA/2J x C3H.NBSn)F1 parabiosis, this does not always occur (Table 4) and red blood cell chimerism can remain in the F1 partner after 100 days in parabiosis with DBA/2J. It must be emphasized that an apparently complete parental red cell takeover is the usual result of parabiosis in this strain combination also, although, as noted here, exceptions sometimes occur. In two of these pairs, which were chimeric for GPI isozymes when the F1 partners were tested at 113 and 124 days after joining, tests at more than 300 days after parabiosis revealed them to be no longer chimeric for GPI. A possible factor is that the takeover process, initiated at parabiosis, took an unusual length of time to reach completion in these pairs. Whether this is really characteristic of the DBA/2J with (DBA/2J x C3H.NBSn)F1 strain combination would require more GPI tests than were done here. In the few long term survivors of the DBA/2J with (DBA/2J x C3H.SWSn)F1 strain combination, no such red blood cell chimerism was found in the F1 partner.

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Table 4. Presence of Red Blood Cell Chimerism Detectable by Starch Gel Electrophoresis in Parabionts of Various Strain Combinations

DBA/2J(H-2d) + (DBA/2J) x C3H/HeJ)F1(H-2d/H-2k)	0/22
DBA/2J(H-2d) + (DBA/2J x C3H.NBSn)F1(H-2d/H-2p)	5/18
DBA/2J(H-2d) + (DBA/2J x C3H.SWSn)F1(H-2d/H-2b)	0/3

Occurrence of red blood cell (RBC) chimerism, detectable by starch gel electrophoresis of glucose-phosphate isomerase isozymes from peripheral RBC lysates, from parabionts of various strain combinations. Absence of RBC chimerism indicates an apparently complete takeover of F1 red cell system by cells from the parental partner within the limits of the method.

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b) Intra-H-2

Using the C3H.OH and C3H.A strains, it is possible to ask what effect substitution of only part of the H-2 complex has on long term survival. The H-2 haplotype of C3H.OH differs from the DBA/2J haplotype only at the D end of the H-2 complex. The C3H.A haplotype differs from that of DBA/2J at the K through I-E regions of the H-2 complex (see Table 1), presenting the greater histoincompatibility as shown by the experiments of others (Rychlitova, et. al., 1970, Klein, 1972, McKenzie and Snell, 1973). When the F1 target haplotype was derived from C3H.OH or C3H.A, the proportions of parabiosed animals surviving beyond 100 days were 24/29 (83%) and 21/36 (58%) respectively. This should be compared with the 44% survival seen when an entire H-2 haplotype is foreign (Figure 8). These results are consistent with the general observation that differences in the left hand end of the H-2 complex are more potent transplantation barriers than those in the right hand end (Nisbet and Edwards, 1973, Klein, 1975). It is interesting to note that the C3H.OH and C3H.A parabiont mortality figures (about 17% and 42% respectively), if combined, roughly equal the mortality of the C3H/HeJ parabionts (about 56% in Figure 8).

Another consequence of the strength of the haplotype barrier involves the takeover reaction. When the target haplotype differed from the parental haplotype at the left hand end of the H-2 complex (DBA/2J plus (DBA/2J x C3H.A)F1

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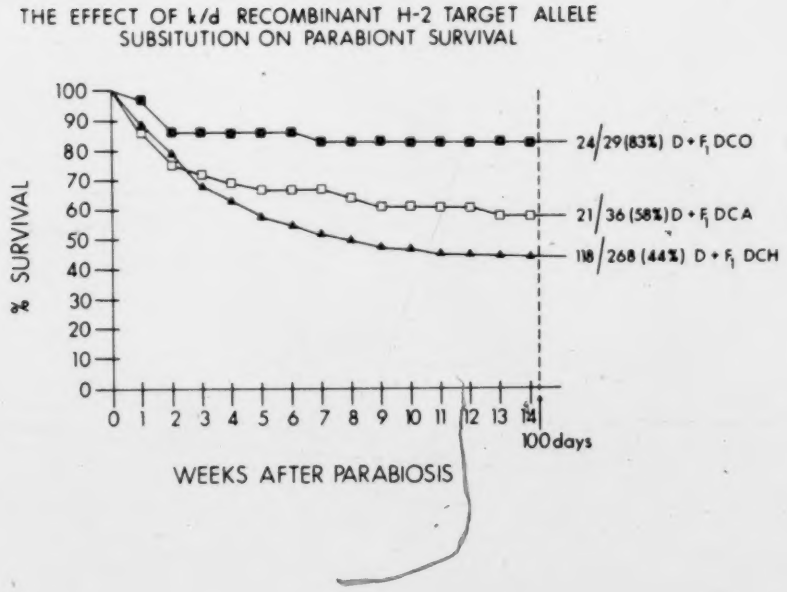

Figure 8.

The effect of k/d recombinant H-2 target allele substitution on parabiont survival to 100 days. Cumulative survival up to 100 days is shown for three strain combinations of parent (DBA/2J) plus F1 hybrid parabioses. DBA/2J (H-2d) plus (DBA/2J x C3H/HeJ)F1 (H-2d/H-2k) serves as the reference strain combination. C3H.A (H-2a) and C3H.OH ($H\text{-}2^{o2}$) carry H-2 haplotypes which are derived from recombinant events between H-2d and H-2k; for the suspected point of crossover, see Table 1. This graph is based on survival data from the number of each type of parabiont made (the denominator in the fraction at the end of each curve). The mortality within each strain combination combines all causes of death except the very few most obvious technical accidents; the data from these were not included. Thus these curves are probably slight underestimates: D + F1DCO = DBA/2J + (DBA/2J x C3H.OH)F1 parabionts: D + F1DCA = DBA/2J + (DBA/2J x C3H.A)F1 parabionts; and D + F1DCH = DBA/2J + (DBA/2J x C3H/HeJ)F1 parabionts.

parabionts), all pairs examined at times considerably beyond 100 days showed complete parental hemopoietic takeover (6/6). In contrast, when the two haplotypes differed only at the right hand end of H-2, (DBA/2J plus (DBA/2J x C3H.OH)F1 parabionts), there was no takeover of the F1 hybrid by the parental cells in any of 18 pairs tested, at times ranging from 74 to 513 days after parabiosis. Within a given pair, both partners showed comparable degrees of GPI chimerism but there was variation between pairs, and many pairs showed degrees of chimerism clearly different from a 1:1 mix (Figures 9a and b). Thus the K end may play a crucial role as a target antigen in the takeover reaction.

c) Parental Polycythemia and Hybrid Anemia are Missing in Parabionts Incorporating Partial H-2 Differences

A common observation following parent plus F1 hybrid parabiosis is that the parental partner becomes polycythemic and the F1 partner becomes anemic (Eichwald, et. al., 1959, 1960, 1963). A dramatic polycythemia-anemia syndrome in DBA/2J plus (DBA/2J x C3H/HeJ)F1 parabionts, which disappears in those pairs which survive, was noted earlier (Figure 5). There was no such wide divergence of hematocrits in DBA/2J plus (DBA/2J x C3H.NBSn)F1 pairs, (Figure 7) although the differences between parent and F1 were statistically significant ($P < 0.05$) at 3 weeks.

Figure 10 (a and b) shows the hematocrit profiles of DBA/2J plus (DBA/2J x C3H.A)F1 parabionts and DBA/2J plus

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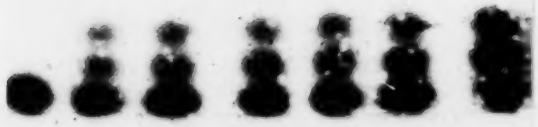

DBA DBA* #1 F1* #1 DBA* #2 F1* #2 1:1 MIX DBA: F1 Normal F1

Figure 9.

Starch gel electrophoresis for isozymes of glucose phosphate isomerase from lysates of RBC from four representative DBA/2J (H-2d) plus (DBA/2J x C3H.OH)F1 (H-2d/H-2^{o2}) parabionts. 'DBA*' refers to the parent partner; 'F1*' refers to the parabiosed (DBA/2J x C3H.OH)F1 partner. Fourteen other parabionts of this strain combination gave very similar data. The two gels show the retention of hybrid derived enzyme bands in lysates from the parental parabiont partners as well as in lysates of the F1 hybrids from the same pairs. Lysates from the parabionts in slab a were made 434 days after parabiosis; those illustrated in slab b were made 285 days after parabiosis. The 1:1 mix is an artificial mixture of lysates from normal DBA/2J and normal unparabiosed (DBA/2J x C3H/HeJ)F1 (GPI-1a/GPI-1b, identical with (DBA/2J x C3H.OH)F1 on starch gels).

Figure 9b. See legend to Figure 9a.

Normal F1 1:1 MIX DBA: F1 F1* #3 DBA* #3 F1* #4 DBA* #4 DBA

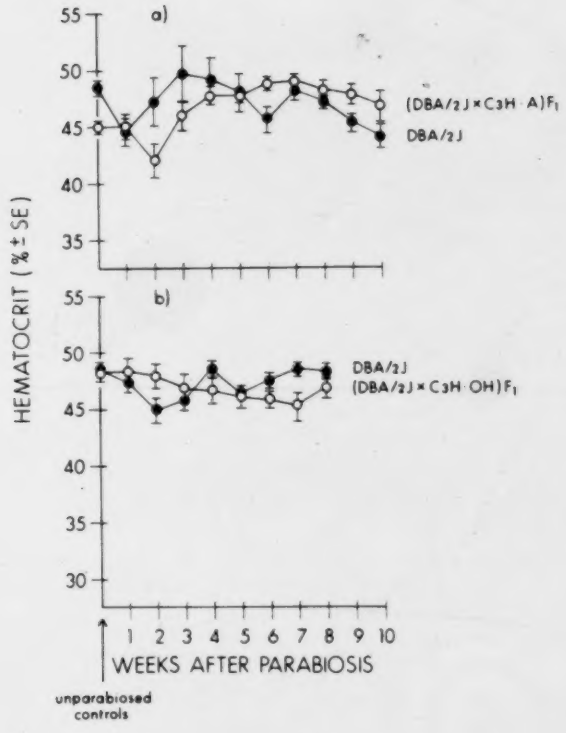

Figure 10.

(a) Changes in hematocrit values with time after parabiosis for DBA/2J plus (DBA/2J x C3H.A)F1 parabionts. Difference at two weeks is statistically significant ($P < 0.05$). Values plotted are the % hematocrit $\pm$ S.E. Each point is the mean of data from at least 6 parabiont partners of the indicated strain.

(b) Change in hematocrit values with time after parabiosis for DBA/2J plus (DBA/2J x C3H.OH)F1 parabionts. Difference at two weeks is significant ($P < 0.05$). Values plotted are the % hematocrit $\pm$ S.E. Each point is the mean of data from at least 9 parabionts of the indicated strain.

(DBA/2J x C3H.OH)F1 parabionts, respectively. There is no dramatic initial divergence of parental hematocrit values from F1 hybrid values in either strain combination, although the difference at 2 weeks after parabiosis is statistically significant ($P < 0.05$, DBA/2J vs. F1 for both strain combinations). The slight dip in hematocrits one to two weeks after parabiosis is seen in syngeneic parabionts (DBA/2J plus DBA/2J) as well and does not reflect an immune reaction (data not shown). Figure 10 indicates that with only partial H-2 differences, there is no drastic alteration in the proportion of F1 RBC mass to parental red cell mass, as is seen in most other parent-F1 hybrid parabioses (Eichwald, et. al., 1963) including the DBA/2J plus (DBA/2J x C3H/HeJ)F1 parabionts (Figure 5).

2. Non-H-2 Background Genes do not Influence Long Term Parabiont Survival When DBA/2J is the Responding Partner to H-2k Alloantigens

The unusual survival of the DBA/2J plus (DBA/2J x C3H)F1 hybrids in parabiosis might be influenced by the non-H-2 genetic background provided by the C3H strains. To evaluate this, crosses were made between DBA/2J and two lines of CBA: CBA/CaJ (derived from CBA/H) and CBA/J. These two lines are similar in many respects to C3H/HeJ (all are H-2k), but known differences include the Mls locus (Festenstein 1974, see Table 1), and at least one specificity at the K end of H-2: CBA/CaJ lacks the H-2.8 specificity present in C3H/HeJ and CBA/J (Green and Kaufer,

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1965). Parabionts were then made between DBA/2J and both CBA hybrids. Their survival curves (Figure 11) show that there was no statistically significant difference in survival at 100 days for either DBA/2J with (DBA/2J x CBA/CaJ)F1 parabionts (38%), or DBA/2J with (DBA/2J x CBA/J)F1 parabionts (33%) when compared to the reference strain (DBA/2J with (DBA/2J x C3H/HeJ)F1, 44%). These observations provide no evidence for the influence on parabiont survival of those non-H-2 genes by which C3H/HeJ, CBA/CaJ, and CBA/J differ, when DBA/2J is the responding parental partner. However these results do not exclude the possible involvement on parabiont survival of other non-H-2 genes which these three strains may share.

GPI analysis of RBC lysates from DBA/2J plus (DBA/2J x CBA/CaJ)F1 parabionts, tested at 241 or 314 days after parabiosis, showed a complete parental takeover of the F1 partner. An identical result was observed in four DBA/2J plus (DBA/2J x CBA/J)F1 pairs, tested at 175 or 200 days following parabiosis (data not shown). Thus, the DBA/2J plus (DBA/2J x CBA)F1 parabionts show very similar long term survival, and an identical takeover reaction of the F1 partner, to the conventional DBA/2J plus (DBA/2J x C3H/HeJ)F1 strain combination.

With respect to the two types of CBA, we next asked what effect changing the responder type had on parabiont survival. To this end, BALB/cCR (H-2d) mice were parabiosed

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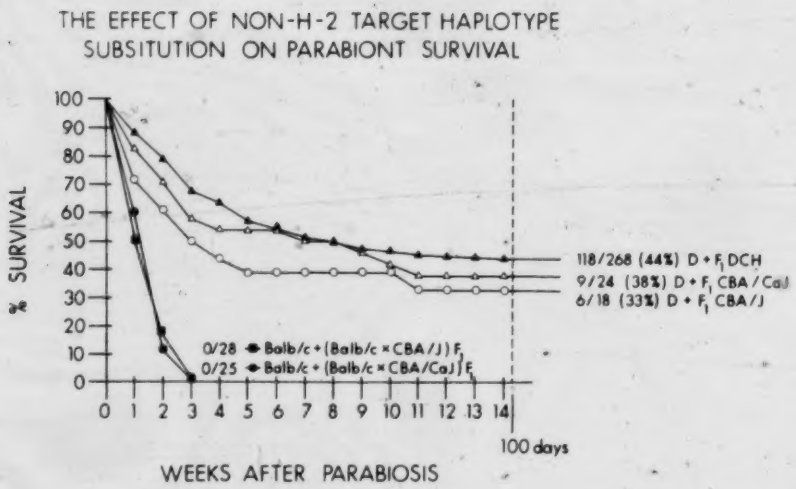

Figure 11.

The effect of non-H-2 haplotype substitution on parabiont survival to 100 days. Target: Cumulative survival up to 100 days is shown for three DBA/2J plus (DBA/2J x H-2K)F1 hybrid strain combinations. DBA/2J (H-2d) plus (DBA/2J x C3H/HeJ)F1 (H-2d/H-2K) serves as the reference strain combination. None of the survival percentages at 100 days were significantly different from the others ($P > 0.05$ for all two-way comparisons): D + F1DCH = DBA/2J + (DBA/2J x C3H/HeJ)F1 parabionts; D + F1CBA/CaJ = DBA/2J + (DBA/2J x CBA/CaJ)F1 parabionts; and D + F1CBA/J = DBA/2J + (DBA/2J x CBA/J)F1 parabionts. Responder: Cumulative survival is shown for two strain combinations in which DBA/2J was replaced by BALB/c (also H-2d) as the responding parental partner. Out of the total of 53 pairs, none survived beyond 3 weeks. As in Figures 6 and 8, mortality was calculated by including all causes of death except the most obvious technical accidents.

to either (BALB/cCR x CBA/CaJ)F1 or to (BALB/cCR x CBA/J)F1 partners. As shown in Figure 11, both parabiont combinations proved lethal within three weeks. This could be the result of the known strong reactivity of BALB/c, compared to DBA/2J, to Mls-locus differences (Festenstein, 1973), although numerous other non-Mls-locus background genes could also be involved.

Finally, (DBA/2J x BALB/cCR)F1 hybrids and (DBA/2J x C3H/HeJ)F1 hybrids were joined in parabiosis to investigate whether the unusual survival seen with DBA/2J responders is dominant over the 0% survival observed with BALB/cCR responders. Of 27 such parabionts made, all were ultimately unsuccessful. 7/27, however, survived beyond 3 weeks; the longest survivor lived for 7 weeks. Thus, while the survival profile for these parabionts was better than for BALB/cCR plus (BALB/cCR x H-2k)F1 pairs, the presence of BALB/cCR in the responder partner resulted in the failure of all of the parabiont combinations tried.

Thus a change in all or part of the F1 H-2 complex (with the reservations noted earlier for the use of congenic mice for this type of analysis) results in major effects on parabiont survival, hematocrit profile, and hemopoietic takeover. Changing the non-H-2 background of the F1 partner does not significantly affect survival or takeover (hematocrits were not analyzed). Substituting a different H-2d parental strain (BALB/cCR), even including it in half a

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dose (in a (BALB/cCR x DBA/2J)F1 hybrid partner), when responding to a (DBA/2J x H-2K)F1 hybrid partner, eliminated survival altogether (Drell and Wegmann, 1979).

III. Immunologic Status of Successful Parabionts

The demonstration by Shaw, et. al., (1974) that spleen lymphocytes from DBA/2J with (DBA/2J x C3H/HeJ)F1 parabionts retained in vitro mixed lymphocyte responsiveness to C3H/HeJ alloantigens indicated that immunologically these mice were not tolerant, merely unresponsive in vivo. This distinction is an important one especially in recent years when it has become apparent that observations of unresponsiveness can be accounted for in a number of ways (see Introduction). Since parabionts were unresponsive in vivo but responsive in vitro, various experiments designed to further characterize this apparent paradox and throw light on the mechanisms involved were begun.

1. Cell Mediated Cytotoxicity

The MLR measures proliferative ability; to test effector ability, ^{51}Cr release assays, using PHA-stimulated C3H/HeJ targets (see Materials and Methods), were performed. When spleen cells were removed from the parabionts and tested immediately, neither the parent nor the F1 hybrid were capable of giving direct killing by this assay (Table 5). Spleen cells were from mice in parabiosis for between 121 and 177 days.

Only one Effector:Target ratio was tested (100:1) but

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Table 5. Percentage of direct killing of PHA stimulated-^{51}Cr labeled C3H/HeJ target cells by parabiont* and presensitized normal spleen cells (0% killing is defined as that given by unsensitized normal spleen cells).

	Experiment #							
Spleen Cell Donor	1	2	3	4	5	6	7	8
DBA*	1.8	0.9	3.9	-0.4	-7.2	3.8	0.2	2.7
F1*	9.8	-10.0	3.6	1.2	-6.2	-6.9	-0.4	0.7
DBA Presensitized to C3H	--	--	75.8	53.9	44.2	51.4	28.7	63.8

at this ratio, the presensitized normal DBA/2J spleen cells gave very good specific ^{51}Cr release. Although other allogeneic targets were not tried in these experiments it is clear that sensitized anti-C3H/HeJ cells were not present, in active form, in parabiont spleens.

When the parabiont parental and F1 hybrid spleen cells were co-cultured, for 5 days in Marbrook chambers with irradiated C3H/HeJ stimulator cells, and then an assay for the presence of cytotoxic cells performed, killer lymphocytes were present (Table 6).

What is immediately apparent from these data is that parabiont parental spleen cells gave rise, upon 5 day <u>in vitro</u> development (essentially a 5 day MLR) to a degree of cytotoxicity against C3H/HeJ targets as good as normal DBA/2J spleen cells. The F1 parabiont spleens developed markedly less cytotoxicity against C3H/HeJ. Dr. S.M. Phillips (University of Pennsylvania School of Medicine,

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Table 6. Percentage of killing of PHA stimulated ^{51}Cr labeled C3H/HeJ target cells by parabiont* and normal control spleen cells after 5 days in vitro with irradiated C3H/HeJ spleen cells.

Spleen Cell Donor	Experiment # 1	2	3	4	5	6	7	8
DBA*	92.4	50.2	54.3	34.8	47.8	30.1	36.9	39.0
F1*	7.2	23.9	21.7	--	23.3	12.7	--	5.5
DBA normal	91.9	47.2	54.1	44.2	51.4	28.7	63.8	53.7
F1 normal	--	--	--	2.8	-5.3	17.7	4.3	-4.4

Philadelphia, Pennsylvania) tested four pairs of our parabionts and obtained similar data (personal communication). Thus in addition to retaining MLC reactivity, parabiont spleen cells, particularly from the parental partner, can respond in vitro to C3H/HeJ stimulator cells by giving rise to cytotoxic effector cells (Wegmann and Drell, 1975).

A small number of experiments were done in which parabiont spleen cells were cultured for 1 or 3 days without stimulation by C3H/HeJ alloantigen; this was to determine if cytotoxicity would reemerge subsequent to the hypothetical in vitro elution of inhibitory material ("blocking factors"). No cytotoxicity of ^{51}Cr-labeled C3H/HeJ target cells was seen after such pre-culture (Table 7) suggesting that the cytotoxicity seen after 5 day in vitro culture was de novo, rather than the expression of a pre-existing but blocked activity.

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2. Graft-Versus-Host Reactivity

Table 7. Percentage of killing of PHA stimulated ^{51}Cr labeled C3H/HeJ target cells by parabiont* spleen cells cultured for 1 or 3 days in the absence of stimulation by alloantigen. Data are % specific ^{51}Cr released.

Spleen Cell Donor	Experiment # 1	2	3
1 day culture			
DBA*	0.0	0.0	--
F1*	3.2	0.0	--
DBA presensitized to C3H	50.5	64.7	--
3 day culture			
DBA*	3.1	5.3	1.5
F1*	--	1.0	--
DBA presensitized to C3H	60.2	78.3	81.7

Figure 12 presents the results of injecting parabiont spleen cells into the rear footpads of F1 hybrids syngeneic to the F1 partner of the parabiosis. With spleen cells from either partner, from both the medium- and high-survivor strain combinations, differential lymph node enlargement results. This enlargement is appreciably greater than that in the negative controls, in which syngeneic F1 cells were injected into both footpads.

The GVH reactivity of parabiont DBA/2J cells generally

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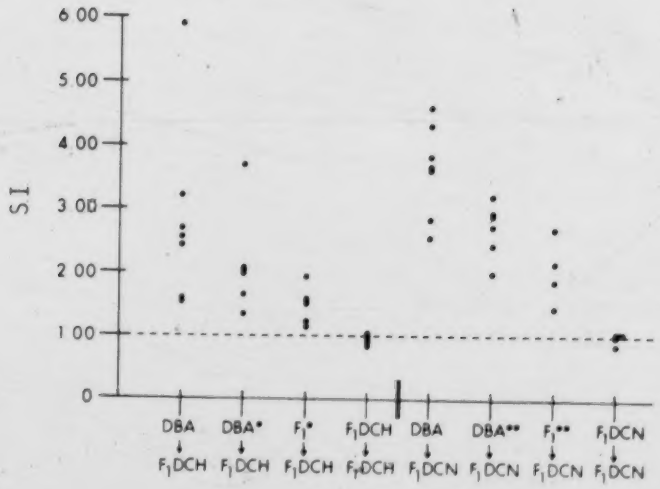

Figure 12.

Popliteal lymph node enlargement ratios of various types of F1 hybrids 7 days after injection of spleen cells from normal DBA/2J, DBA/2J or F1 parabionts, or syngeneic F1s. Each point represents the antilog of the mean of the log-transformed ratios from at least 5 recipients (at least 3 in the F1DCH <n1> -> FIDCH group). A stimulation index (S.I.) of 1.00 indicates no enlargement of the experimental lymph node over the control lymph node. F1DCH -> F1DCH and F1DCN -> F1DCN indicate groups of recipients (negative controls) receiving syngeneic normal F1 spleen cells in both footpads. DBA* and F1* are the parental and F1 partners, respectively, of a DBA/2J with (DBA/2J x C3H/HeJ)F1 (F1DCH) parabiosis. DBA** and F1** are the parental and F1 partners, respectively, of a DBA/2J with (DBA/2J x C3H.NBSn)F1 (F1DCN) parabiosis.

exceeds that of the F1 partners' cells. The reason for this is unknown, but a similar phenomenon was observed in cytotoxicity assays using parabiont spleen cells.

The data in Figure 13, showing parabiont GVH reactivity in F1 hybrids carrying an unrelated H-2 haplotype, are less extensive, since not all parabiont spleens contained enough cells to test in both specific and third-party F1 recipients. Even though only one data point is available for (DBA/2J x C3H.NBSn) F1 parabiont (F1DCN) in third-party (DBA/2J x C3H.SWSn)F1 recipients (F1DCS), that single point and the others in Figure 13 clearly establish that long-term surviving parabionts are not nonspecifically immunosuppressed, as has been reported in other GVH situations (Howard and Woodruff, 1961; Lapp and Moller, 1969); rather, the reactivity of their spleen cells is comparable to that of normal DBA/2J spleen cells in the third-party F1 hybrid recipients. Very similar data (not shown), indicating that DBA/2J plus (DBA/2J x C3H.A)F1 parabionts also retained GVH reactivity were also accummulated (Drell and Wegmann, 1977).

3. In Vivo Tumor Clearance Studies

   a) Reactive Cells to "Tolerated" Alloantigens Persist After Long Term Parabiosis and Will not Inhibit Normal Cell Responses

In order to test the potential reactivity of parabiont cells or serum against H-2k transplantation antigens as well

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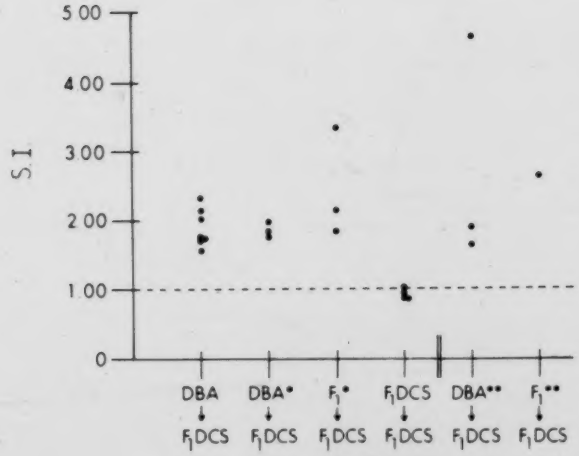

Figure 13.

Popliteal lymph node enlargement ratios of various types of F1 hybrids 7 days after injection of spleen cells from normal DBA/2J, DBA/2J or F1 parabionts, or syngeneic F1s. Each point represents the antilog of the mean of the log-transformed ratios from at least 5 recipients. A stimulation index (S.I.) of 1.00 indicates no enlargement of the experimental lymph node over the control lymph node. F1DCS -> F1DCS indicates recipients (negative controls) receiving syngeneic normal F1 spleen cells in both footpads. DBA*, F1*, DBA**, F1** are as described in the legend to Figure 12. F1DCS is a (DBA/2J x C3H.SWSn)F1 recipient.

as the possible suppressive influence of parabiont cells on normal DBA/2J reactivity to H-2K, an assay system was needed in which parabiont cells or serum could be transferred and tested. This was achieved with an adoptive transfer system using irradiated (850 R) DBA/2J recipients restored with parabiont or normal DBA/2J spleen cells and injected i.p. 5 days later with the H-2K tumor RI, prelabeled in vivo with ^{131}I-iododeoxyuridine (^{131}I-IUdR, henceforth referred to as ^{131}I-RI). Retention of radioactivity was followed by daily whole body counting of the injected mice.

For these experiments, we capitalized on the clear difference in the rate of clearance of the ^{131}I-RI between normal DBA/2J mice, and DBA/2J mice immunized 5 days earlier to H-2K alloantigens. In the immunized group, tumor rejection was very rapid (Figure 14 curve B) when contrasted to the rate of tumor cell death in the nonimmune group (Figure 14 curve A).

For the next experiments, it was important to determine that neither a response to tumor associated antigens nor any radioresistant contribution by the irradiated recipients of adoptively transferred cells were significant factors in the clearance of the ^{131}I-RI. This was tested by adoptively transferring spleen cells from fetal liver chimeric mice tolerant to C3H/HeJ alloantigens. The fetal liver chimeras were made by injecting 10^7 DBA/2J 10-13 day fetal liver cells into 900R irradiated (DBA/2J x C3H/HeJ)F1 mice

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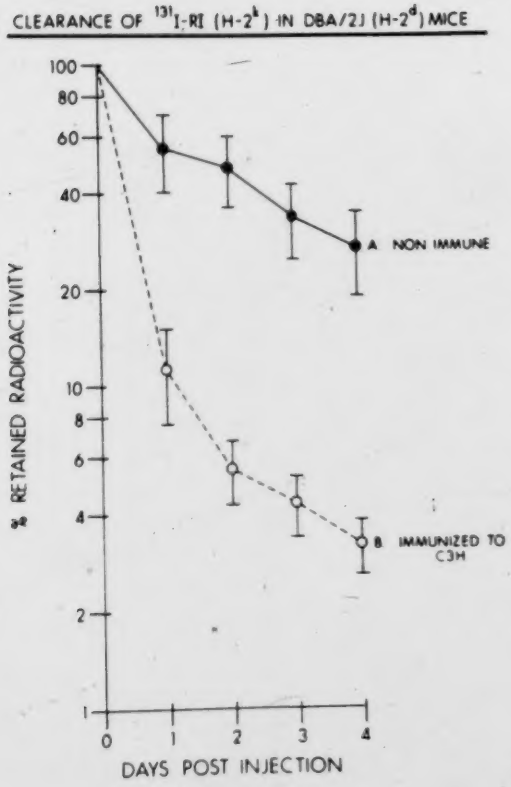

Figure 14.

Clearance of 10^6 ^{131}I-RI (H-2k) tumor cells (approx. 50,000 cpm/mouse on day 0) in normal DBA/2J (H-2d) mice unimmunized (curve A) or presensitized 5 days earlier by an i.p. injection of 10^7 1200R irradiated C3H/HeJ (H-2K) spleen cells (curve B). Points given are the mean $\pm$ SD of at least 5 mice/group.

approximately 16 months before use. These fetal liver chimeras share the immunological characteristics of bone marrow chimeric mice (Sprent, et. al., 1975, and Drell and Wegmann, unpublished observations). When tested in adoptive transfer, recipients of 10^7 spleen cells from DBA/2J into (DBA/2J x C3H/HeJ)F1 fetal liver chimeras could not be immunized with C3H/HeJ spleen cells to rapidly clear the ^{131}I-RI tumor. When spleen cells from DBA/2J into (DBA/2J x C3H.NBSn)F1 fetal liver chimeras, specifically tolerant to H-2p alloantigens, were adoptively transferred to DBA/2J mice and exposed to C3H/HeJ spleen cells, these recipients showed rapid clearance of the ^{131}I-RI. On day 3 after transfer and sensitization to H-2k, recipients of cells from the H-2k - tolerant chimeras showed 49.3% ± 6.3 retention of the ^{131}I-RI, while similarly treated recipients of H-2p - tolerant cells showed 4.8% ± 0.6 retention of the labeled cells, equivalent to 89.7% tumor killing. Nonimmune DBA/2J controls showed 46.5% ± 5.8 retention and immunized DBA/2J controls showed only 5.8% ± 0.8 retention (87.5% tumor killing). Thus the recipients of cells known to be unresponsive to H-2k did not show any response after sensitization. This established that the destruction of the tumor was due to the effects of the transferred cells and not to a response to tumor associated antigens or to any radioresistant host reactivity.

Using this same adoptive transfer approach, we tested parabiont spleen cells. 10^7 spleen cells, pooled from the

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DBA/2J and F1 partners of two to three parabionts, were injected i.v. into 850R irradiated DBA/2J recipients. Half of these recipients (5 mice) were then immediately injected with C3H/HeJ spleen cells; the other half were not. Five days later, 10^6 ^{131}I-RI were injected i.p. and whole-body monitoring begun. Control groups were 850R DBA/2J restored with 10^7 normal DBA/2J spleen cells, with or without sensitization with C3H/HeJ spleen cells. Figure 15 shows the results of one such experiment, representative of 3 experiments done.

When presented with C3H/HeJ spleen cells, parabiont spleen cells responded as well as normal DBA/2J cells. (Compare curves C and D in Figure 15). When not restimulated at the time of adoptive transfer, no rapid tumor clearance was seen, indicating that parabiont spleen cells surviving at the time of test challenge did not express effector activity against C3H/HeJ alloantigens. These results agree with the earlier finding that parabiont cells could be stimulated <u>in vitro</u> against C3H/HeJ, but did not express any pre-existing reactivity. Two analogous experiments transferring 5 x 10^7 parabiont cells gave identical results (data not shown). This made it unlikely that at the 10^7 cell dose, a cell type capable of suppressing the tumor killing had been diluted out.

The hypothesis that parabiont spleen cells could inhibit the development of a response to the ^{131}I-RI by

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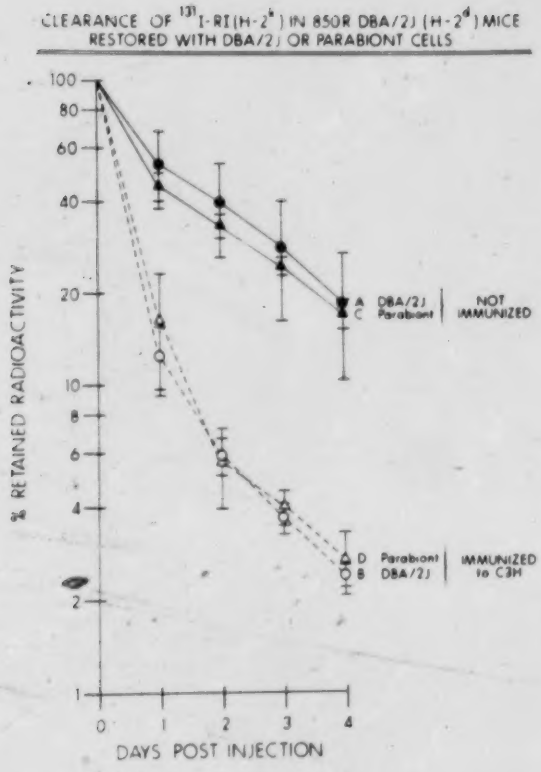

Figure 15.

Clearance of 10^6 ^{131}I-RI (H-2k) tumor cells from DBA/2J (H-2d) mice irradiated with 850R and restored with 10^7 normal DBA/2J spleen cells (curves A and B) or 10^7 parabiont spleen cells from both partners (curves C and D). Half of the mice were then stimulated (5 days before tumor injection) by the injection, i.p., of 10^7 irradiated (1200R) C3H/HeJ (H-2k) spleen cells.(curves B and D). Each curve is the clearance rate of the tumor from at least 5 mice (means $\pm$ S.D.)

normal DBA/2J spleen cells was tested by mixing 10^7 parabiont spleen cells with 10^7 normal DBA/2J spleen cells and adoptively transferring them to 850R DBA/2J recipients. Figure 16 shows that there was no reduction in the anti-RI response of this mixture after stimulation with C3H/HeJ spleen cells, compared to the response of normal DBA/2J spleen cells. Thus parabiont cells did not interfere in the sensitization of the normal DBA/2J cells to C3H/HeJ alloantigens. In summary, parabiont spleen cells, alone or mixed with normal DBA/2J spleen cells, did not differ from normal DBA/2J cells in their responsiveness to C3H/HeJ alloantigens (Drell, et al., 1979a, b).

b) Parabionts Show Rapid Clearance of the RI Tumor In Situ

The previous experiments confirmed the potential for reactivity of parabiont cells when removed from the parabionts. We next analyzed this potential within the parabionts themselves. Our initial experiments used parabiont mice which were surgically separated 7 days, or only 1 day before being re-exposed to H-2K alloantigens on lymphoid cells. Five days after priming, each mouse was challenged with 10^6 ^{131}I-RI. Controls included separated and immunized DBA:DBA parabiont mice, as well as immunized and normal DBA/2J mice. Separated syngeneic (DBA/2J:DBA/2J) parabionts, when immunized, did not differ from immunized normal DBA/2J mice; thus, this control was not repeated. In contrast to the adoptive transfer experiments, the results

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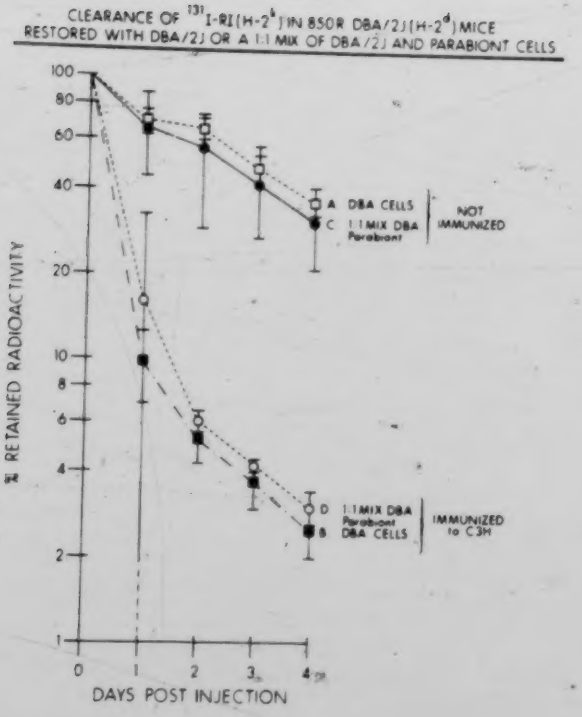

Figure 16.

Clearance of 10^6 ^{131}I-RI (H-2k) tumor cells from DBA/2J (H-2d) mice irradiated with 850R and restored either with 10^7 normal DBA/2J spleen cells (curves A and B) or a 1:1 mixture of normal DBA/2J spleen cells and pooled parabiont spleen cells (10^7 of each, Curves C and D). Curves B and D represent restored DBA/2J mice stimulated at the time of restoration by an i.p. injection of irradiated (1200R) C3H/HeJ (H-2k) spleen cells. Means $\pm$ SD are given for at least 4 mice per group. The large standard deviation for primed mixed parabiont and normal cells on day 1 after tumor inoculation, is due to a single animal with a relatively slower clearance rate on the first day but which, on subsequent days, gave a clearance rate much closer to the three other mice in group D.

indicated that both separated parabiont partners, whether stimulated with H-2K, or not, rapidly cleared the RI tumor (Figure 17 and 18). This accelerated clearance is especially dramatic with the DBA/2J partners (Figure 17); their clearance rate is indistinguishable from the immunized controls. Thus the DBA/2J parabionts behave as though they were already primed to H-2K alloantigens. The immunized F1 partners killed the tumor less rapidly than the immunized DBA/2J partners while in the separated, unstimulated F1 ex-parabionts, accelerated destruction of the tumor was seen in two out of three individuals tested (Figure 18). These results, although more variable, resembled the results from the DBA/2J partners.

It was possible that the release of antigen during separation of the pairs could have led to priming. To test this, intact parabionts were injected with 10^6 ^{131}I-RI cells. On day 3, the parabionts were sacrificed, separated and immediately counted for retained radioactivity. Similarly injected, normal DBA/2J controls were counted daily. The results expressed as the percent of the tumor killed, by seven parabionts in three separate experiments, are shown in Table 8. In both partners, the tumor killing on day 3 was significantly higher than the day 3 value for the unimmunized controls ($P < 0.01$ for each point with respect to control on day 3). Some mechanism present in the intact parabionts caused the accelerated destruction of the labeled cells, albeit not as efficiently as in the immunized

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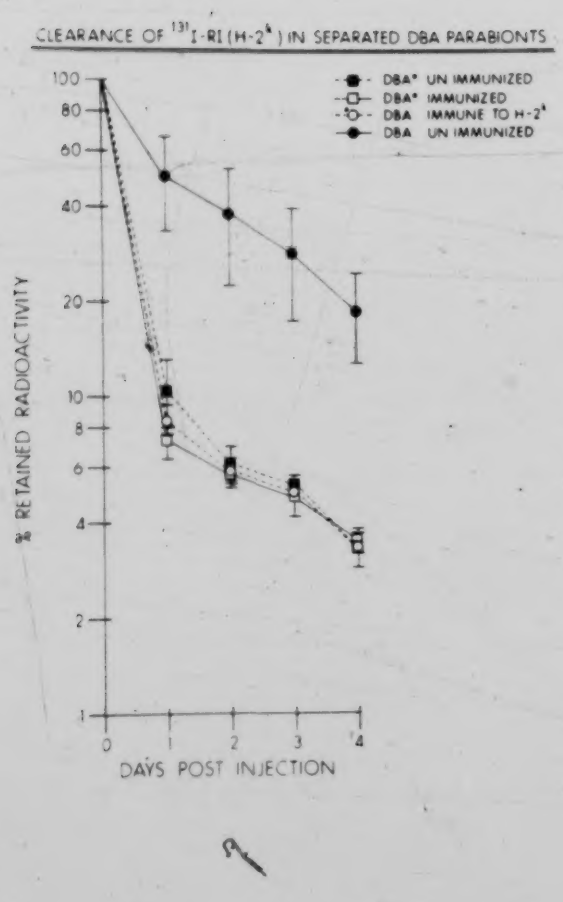

Figure 17.

Clearance of 10^6 ^{131}I-RI (H-2k) tumor cells in DBA/2J partners (H-2d) formerly in parabiosis with (DBA/2J x C3H/HeJ)F1 (H-2d/H-2k) mice. One day after surgical separation, three of the DBA/2J partners were stimulated against H-2k with an i.p. injection of irradiated (1200R) C3H/HeJ spleen cells. Three others were unstimulated; 5 days later, the ^{131}I-RI was injected and daily whole body counting begun. Means $\pm$ SD are given

- normal DBA/2J unimmunized
- normal DBA/2J immunized to C3H/HeJ
- Formerly parabiosed DBA/2J partners, unstimulated
- Formerly parabiosed DBA/2J partners, stimulated against C3H/HeJ

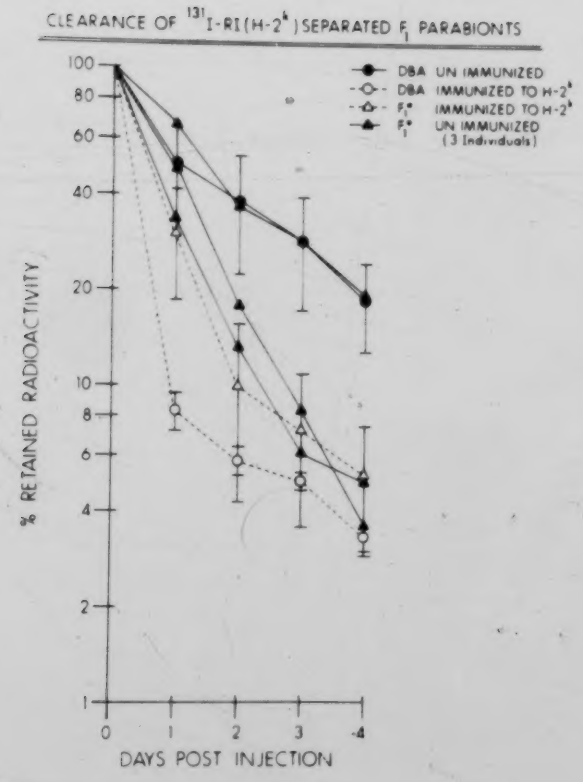

Figure 18.

Clearance of 10^6 ^{131}I-RI (H-2k) tumor cells in (DBA/2J x C3H/HeJ)F1 partners (H-2d/H-2k) formerly in parabiosis with DBA/2J (H-2d) mice, treated as described for DBA/2J partners in legend for Figure 17. Three separated F1 partners, plotted here as individuals (rather than means $\pm$ SD, given for the other groups) were not stimulated against C3H/HeJ). 5 days later, ^{131}I-RI was injected and daily whole body counting begun for all individuals.

—●— normal DBA/2J unimmunized
---○--- normal DBA/2J, immunized to C3H/HeJ
—▲— formerly parabiosed F1 partners, unstimulated (3 individuals)
---△--- formerly parabiosed F1 partners, stimulated against C3H/HeJ

Table 8. Percent of ^{131}I-RI tumor killed on day 3 after I.P. injection into unseparated parabiont partners

	(% retention of ^{131}I ± S.D.)		
	Exp. 1	Exp.2	Exp.3
DBA/2J NON IMMUNE	0(32.3 ± 6.4)	0(44.2 ± 8.4)	0(37.8 ± 3.5)
DBA/2J IMMUNE to C3H	87.3(4.1 ± 0.3)	91.6(3.7 ± 0.4)	91.5(3.2 ± 0.4)

DBA/2J	Pair #1	Pair #2	Pair #3	Pair #4	Pair #5	Pair #6	Pair #7
PARABIONT PARTNER	86.1	84.5	64.7	68.6	84.1	68.8	85.4
F1 PARABIONT PARTNER	75.8	57.9	47.3	69.9	88.9	90.5	87.8

controls. It is worth emphasizing that these parabionts had all survived for longer than 100 days together, were in apparent good health, and had been subjected to no manipulations after parabiosis prior to use in these experiments.

Thus a mechanism exists within the intact parabionts which is effective *in situ* against alloantigens shared by the F1 partner, yet does no apparent harm *in vivo*. Whether circulating antibody could be the explanation for these results was tested next.

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4. Evidence for Antibody

a) Passively Transferred Parabiont Sera Accelerate Tumor Destruction In Vivo

In three separate experiments, sera pooled from several intact parabionts were tested after passive transfer to DBA/2J recipients. Figure 19 shows the results of mixing pooled parabiont serum with the ^{131}I-RI immediately before injection; each of 5 DBA/2J recipients received the equivalent of 0.1 ml serum. In this, the best experiment, the recipients of the parabiont serum cleared the tumor as rapidly as the recipients of 0.1 ml of a high titered anti-C3H/HeJ serum. In two subsequent experiments, however, in which separate pools of DBA/2J and F1 parabiont sera were tested, fewer tumor cells were killed; however, acceleration of tumor destruction was seen with both pools. The controls, DBA/2J recipients of 0.1 ml of normal DBA/2J serum, showed 32.3% ± 6.4 retention of the tumor on day 3, while the recipients of DBA/2J parabiont serum showed 24.9% ± 8.4 retention. The recipients of F1 parabiont serum showed 23.2% ± 6.2 retention. The differences in clearance rates in this experiment, while not statistically significant, may reflect variation in the amount of antibody in individual parabionts. In order to more efficiently use the small amounts of serum available, in vitro tests were done.

b) Parabiont Sera Show in Vitro Complement Dependent Cytotoxic Activity Against H-2k Bearing Target Cells

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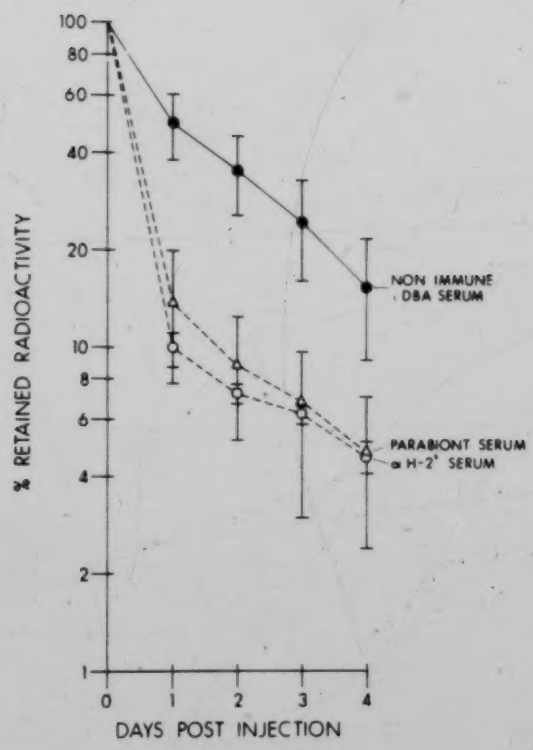

Figure 19.

Clearance of ^{131}I-RI(H-2k) tumor cells from DBA/2J mice given parabiont serum in passive transfer with the tumor. Non-immune mice received 0.1 ml normal DBA/2J serum with the tumor. The other control group received 0.01 ml of a high-titered anti-C3H/HeJ serum with the RI. The mice treated with parabiont serum received 0.1 ml each along with the tumor. In two subsequent experiments, mice receiving smaller amounts of parabiont DBA/2J or F1 serum, showed less dramatic tumor clearance than in this experiment.

Sera from both partners of 8 pairs of mice were tested individually for complement dependent cytotoxicity by dye exclusion against either RI tumor targets or C3H/HeJ spleen cell targets. Figure 20 shows the titration curves of representative sera from four intact parabionts, titered against the RI tumor. Parabiont sera from all four DBA/2J and two of four F1 partners showed similar titers (1:15 - 1:20) against the H-2K target. As seen in Figure 20, most of this cytotoxic titer was lost after pre-treatment of the sera with 2-mercaptoethanol, to disrupt IgM. When parabiont sera were tested against the L1210 tumor (H-2d), no lysis was seen (data not shown).

The titers of F1 parabiont sera showed more variability. In two of the four samples shown in Figure 20, as well as a number of others tested since, the F1 partner showed much weaker antibody activity against H-2K. Maximum lysis did not exceed 50% and occurred only at the lowest dilutions. At least one F1 partner, however, had an antibody titer as great as the titer in its DBA/2J partner. With either partner this titer was largely sensitive to 2-mercaptoethanol treatment, suggesting it was IgM.

c) Parabiont Sera Tested Against C3H Congenic Targets: Specificity for K/I End of H-2K

Sera from parabionts that had shown significant titers against RI tumor targets were tested against a panel of spleen cell targets from C3H congenic (C3H.NB, H-2p, C3H.A,

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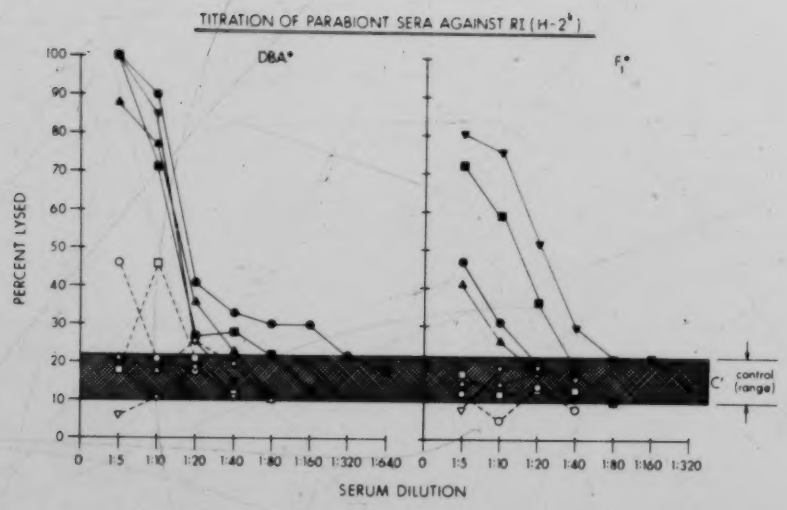

Figure 20.

Titration (by dye exclusion cytotoxicity assay) of DBA/2J (left) and F1 (right) sera from 4 parabionts, tested on RI (H-2k) tumor cell targets. Hatched area covers the range of the complement controls. Dotted lines with open symbols are 2-mercaptoethanol-resistant titers. Each symbol (●, ■, ▲, and ▼) represents one pair of parabionts.

H-2a, and C3H.OH, H-2^{o2}) mice, and from mice of the C3H/HeJ reference line. H-2 genotypes are given in Table 1. In all sera tested, antibody lytic for target cells presenting surface alloantigens determined by the entire H-2k haplotype or the left "half" of the H-2k haplotype (e.g. H-2a, Kk I-Ak I-Bk I-Jk I-Ek) was found (Figure 21). The C3H.OH target was not lysed, nor was C3H.NB, which served as a control for non-H-2 target antigens determined by the C3H genetic background. Thus the activity of parabiont sera is directed against surface antigens determined by loci in the interval from H-2K through H-2I expressed by the H-2k haplotype. Non H-2 antigens contributed by the C3H background do not serve as targets for complement dependent lysis, nor do antigens coded for by the H-2D end loci from the H-2k haplotype.

5. Parabiont F1 Skin Grafts are Rejected in Normal Time by DBA/2J Hosts

Since the circulatory and immune systems of F1 parabiont partners have been taken over by DBA/2J-derived cells, it is expected that in F1 parabiont tissue grafts, all passenger lymphocytes (Billingham, 1971) would be of DBA/2J genotype and might reduce the immunogenicity of these F1 grafts on fresh DBA/2J recipients. Small pieces of flank skin from two F1 parabionts were grafted onto DBA/2J mice, control mice received normal F1 skin, and graft survival times on the coded mice were measured blind (Table 9). F1 parabiont skin was found to enjoy no privilege whatsoever compared to normal F1 skin, when grafted to DBA/2J

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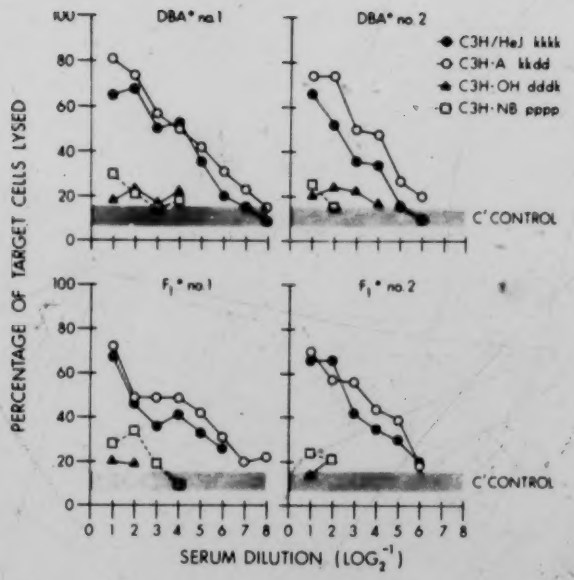

Figure 21.

Titration of two of the same parabiont sera analysed in Figure 20 on spleen cell targets from mice congenic to C3H/HeJ. See Table 1 for H-2 haplotypes. The sera titered here were those in which both the partners showed strong titers against RI targets (■ and ▼ , from Figure 20).

● C3H/HeJ targets (kkkk)
○ C3H.A targets (kkdd)
▲ C3H.OH targets (dddk)
□ C3H/NB targets (pppp)

Hatched area represents the range of values of the complement controls.

recipients; survival times for both types of graft were 12-13 days. F1 parabiont skin was accepted by F1 recipients, however, indicating that it has not lost its capacity to survive transplantation as a result of parabiosis, and had not acquired histocompatibility determinants foreign to normal F1 hybrids (Drell, et al., 1979b).

Table 9. Survival of F1 parabiont skin grafts on fresh DBA/2J recipients

Recipient	Donor	Mean Rejection Time in Days, ± SD, (# of grafts) Exp.1	Exp.2
DBA/2J	normal F1	12.9 ± 0.5 (11)	13.0 ± 1.1 (11)
DBA/2J	parabiont F1	13.5 ± 0.4 (13)	12.1 ± 1.7 (9)
(DBA/2J x C3H/HeJ)F1	parabiont F1	-ND-*	-NR-**(6)
DBA/2J	normal DBA/2J	-NR-**(10)	-ND-*

* ND, Not Done
** NR, No Rejections

IV. Takeover by Cell Injection: Preliminary Work

Since parabiosis results in the takeover of the F1 hybrid partner's lymphoid and erythropoietic systems by parent-derived cells, and immunocompetence to F1 alloantigens is a takeover prerequisite, an investigation was begun to determine whether parental spleen cells alone, injected intravenously into adult F1 hybrids, could effect

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takeover in the absence of parabiosis. Only preliminary results are available. Earlier work in graft-versus-host models (Fox, 1962; Batchelor and Howard, 1965) gave a tentative indication that takeover could be achieved with large numbers of cells alone. Thus varying numbers of DBA/2J spleen cells were given to adult (DBA/2J x C3H/HeJ)F1 mice and the glucose phosphate isomerase phenotypes of the mice were followed by starch gel electrophoretic analysis. In one pilot experiment, the adult F1 hybrid recipients were pretreated with a monoclonal antibody against the H-2K antigen derived from one of its parental strains, prior to injection with cells from the other parental strain.

When a total dose of DBA/2J spleen cells numbering 10^8 or less is injected i.v. into (DBA/2J x C3H/HeJ)F1 adult mice, no detectable change in the pattern of GPI isozymes in RBC lysates results, even months after injection (Table 10). When 5×10^8 cells are injected, altered GPI patterns are evident within four weeks. Of 5 F1 mice injected with this dose of DBA/2J spleen cells, 3 ultimately showed a complete takeover. The relatively short half life of mouse RBC (about 23 days, Russell and Bernstein, 1966) precludes persistence of donor RBC to account for this. Two of these mice, checked more than a year after cell injection, showed complete takeover suggesting that after establishment by cell injection, this result is stable. One of these two showed only partial takeover when tested at 5 months; thus the rate of takeover may not have been uniform under these

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experimental conditions.

Table 10. Production of Takeover Chimera Mice by Parental Strain Spleen Cell Injection of Adult F1 hybrids

Donor Spleen Cells (dose i.v.)	Recipient	No. Mice	Observations on RBC Takeover
DBA/2J (5×10^8)	(DBA/2J x C3H/HeJ)F1	5	Nearly complete takeover at 8 weeks, variable chimerism after that. Complete takeover ultimately in 3/5
DBA/2J (10^8)	(DBA/2J x C3H/HeJ)F1		No takeover
DBA/2J (10^8)	(DBA/2J x C3H/HeJ)F1	5	No takeover
DBA/2J (10^8) 0.25 ml "irrelevent" monoclonal antibody injected i.v. one day before injection of spleen cells	(DBA/2J x C3H/HeJ)F1	5	No takeover
DBA/2J (10^8) 0.25 ml anti-H-2K(k) monoclonal antibody injected i.v. one day before injection of spleen cells	(DBA/2J x C3H/HeJ)F1	6	4 survivors tested at 3 months, nearly complete takeover seen in 4/4
CBA/J (5×10^8)	(BALB/cCR x CBA/CaJ)F1	6	Two survivors tested at 10 months, both showed complete takeover
BALB/cCR (5×10^8)	(BALB/cCR x CBA/CaJ)F1	3	At 9 months, 3/3 showed complete takeover

A complete takeover was achieved in two other strain combinations with 5×10^8 spleen cells. CBA/J cells injected into six (BALB/cCR x CBA/CaJ)F1 recipients and BALB/cCR

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spleen cells injected into three similar F1 hybrids both led to complete takeovers. Of the six CBA/J into F1 chimeras, only two survived to be tested at 10 months; both showed complete takeover. All three of the three BALB/cCR into F1 chimeras survived beyond 9 months and showed complete takeover. The numbers of mice in all three of the groups are much too small to draw any conclusions on the mortality of this procedure. The sole point to be emphasized here is that the injection of a very large number of parental spleen cells into an adult, untreated, H-2 incompatible F1 hybrid can result in a stable, persisting and complete takeover, without mortality.

A final pilot experiment was conducted making use of a hybridoma derived antibody directed against H-2K(k). This antibody is cytotoxic <u>in vitro</u> for H-2K target cells. The purpose here was to see if the takeover reaction would proceed using a smaller dose of DBA/2J spleen cells, known not to be able by itself to cause takeover, if the recipients were pretreated with this antibody. Thus, (DBA/2J x C3H/HeJ)F1 mice were given 0.25 mls of this hybridoma i.v., followed the next day by 10^8 DBA/2J spleen cells i.v. Control groups received either cells alone, or cells one day after the injection of 0.25 mls of another, non-H-2 directed hybridoma. Of 6 F1 hybrids given 10^8 cells and the anti-H-2K(k) hybridoma, 4 were tested at 3 months for GPI isozymes. All 4 showed nearly complete takeover by the injected DBA/2J spleen cells, but traces of F1 bands remained.

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Unfortunately, the survival of these mice was poor and they could not be followed longer. The F1 recipients of either 10^8 DBA/2J cells alone, or of 10^8 cells after the injection of "irrelevant" hybridoma, showed no sign whatsoever of takeover. Despite this being a single, very small experiment, it suggests that a simple, precise pretreatment can markedly affect the dose of parental cells able to replace the circulating red cell system of the F1 recipient. Clearly, an enormous amount of work needs to be done to answer such questions as whether the takeover by cell injection extends to the lymphoid organs such as spleen and marrow (as it does following parabiosis), whether anti-C3H/HeJ immunological reactivity persists, whether antibody is present, and whether there are genetic influences on the degree of success of this process. In short, what needs to be answered is how similar cell injection chimeras are to parabiont chimeras and, eventually, whether cell injection chimeras can serve as a useful model for studying bone marrow replacement biology and therapy.

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DISCUSSION

The characteristics of parabiosed mice can be considered in three parts. 1) A takeover reaction effects the replacement of the F1 lymphoid and erythropoietic systems by parent-derived cells. Parental immunocompetence to the F1 is required for this, and the injection of large numbers of parent type cells alone can mimic this. 2) A limited genetic analysis of survival and takeover in parabiosed mice indicates that, as expected, the H-2 complex is of overwhelming importance and that within H-2, the region delimited by the K and I-E subregions is a stronger stimulus than the D end. 3) Concerning the mechanism(s) that allows the H-2 incompatible partners to survive, the retention of MLR, GVH, and *in vitro* cytotoxic reactivity establishes that deletion-based tolerance is not induced. The persistence of reactivity of parabiont spleen cells upon adoptive transfer and antigenic stimulation, and the absence of any suppressive effect on the reactivity of normal cells, put severe restraints on the liklihood that a suppressor cell is involved. The presence of antibody directed at the H-2 antigens of the "tolerated" F1 partner, and the absence of F1 lymphoid and erythropoietic cells because of the takeover reaction, suggest that enhancement, supplemented by the removal of immunostimulatory cells, may account for these results.

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The takeover reaction encompasses circulating red blood cells and lymphocytes of the spleen and bone marrow. Other cell types may be involved as well. A major proportion of skin immunogenicity may be contributed by Langerhans cells, which are derived from mobile bone marrow precursors (Katz, et. al., 1979). Conceivably, the Langerhans cells of the F1 parabiont skin may be parent derived. Regardless, F1 parabiont skin still remains fully immunogenic (Table 9) at the times tested here (141 days after parabiosis in Exp. 1, 195 days after parabiosis in Exp. 2).

The requirement for parental immunocompetence, and the initial hematocrit divergence in the DBA/2J plus (DBA/2J x C3H/HeJ)F1 pairs, suggest that an initial but limited GVH reaction occurs, triggering the takeover process. The absence of a complete takeover in any of the DBA/2J plus (DBA/2J x C3H.OH)F1 pairs (Fig. 9), the infrequent but occasional persistence of GPI chimerism in some DBA/2J plus (DBA/2J x C3H.NB)F1 pairs (Table 4), and the absence of a dramatic hematocrit divergence in those strain combinations not incorporating full H-2 differences (Fig. 10), or incorporating the apparently weaker H-2p stimulus (Fig. 7), all suggest that the strength of the initial GVH reaction can determine the extent of the takeover reaction. GVH reactions vary in strength with immunogenetic disparity (Grebe and Streilein, 1976) but the popliteal lymph node test, which is strongly positive with DBA/2J spleen cells in (DBA/2J x C3H.NB)F1 recipients (Fig. 13) may not be

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sensitive enough to reflect this. Alternatively, the H-2p haplotype might be a strong stimulus for a GVH reaction but other modifying influences may account for the higher survival rate of parabionts and the less-than-total takeover.

In the complete absence of an antigenic difference that the "responding" partner can recognize, which was the case in the bone marrow chimera plus normal F1 parabionts, no takeover occurs and an apparently balanced 1:1 mix of the two blood systems results (Fig. 3). The genetic analysis of this takeover is limited by the congenic mice available but a K plus I region difference seems a minimum requirement for a complete takeover; it would be very interesting to know if an I subregion difference alone would be sufficient, but the C3H congenics to test this do not exist.

The unusual survival of DBA/2J plus (DBA/2J x C3H.NB)F1 pairs, with a moderate frequency of persistent chimerism, is hard to explain; perhaps the H-2p haplotype is less immunostimulatory to H-2d than is the H-2K haplotype, although a look at the chart of H-2 specificities (Klein, 1975) makes this hard to judge. The absence of a hematocrit divergence suggests that there may be less of an initial GVH reaction, but others have observed that an H-2 difference and the anemia-polycythemia do not always correlate (Eichwald, et. al., 1963; Leonard, 1964). Anemia-polycythemia as a symptom of parabiosis intoxication may

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follow from genetic differences other than H-2.

The genetic information encoded in the H-2 complex of the F1 is of central importance to the outcome of parabiosis judged by both survival and takeover. Figure 6 illustrates that changes in the entire "target" H-2 haplotype of the F1 partner, with minimal known changes in other non-H-2 regions, can drastically affect the outcome. As noted before, (Results, section II.1.A) conclusions based on the use of congenic mice must not be overinterpreted; what happens in parabiosis when C3H.NB is the target versus what happens when C3H.SW is the target is probably due to the known differences, all within the H-2 complex, between these two strains. Yet other less easily discerned differences may have important influences and it is really only by analogy to other transplantation studies with congenics (Klein, 1975; Snell, et. al., 1976) that we can argue with any assurance that the observed differences in this system are, in fact, due to H-2 substitutions.

In addition to the effect of entire H-2 haplotype substitutions, partial H-2 haplotype substitutions detectably influence parabiont survival and takeover. When C3H.A provides the immunogenic stimulus, the results closely resemble those seen when C3H/HeJ provides this stimulus. Survival (Figure 8) is comparable and for this sample size, not significantly different. Takeover is complete and full GVH reactivity persists (Figure 12, and Drell and Wegmann,

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unpublished data) in both of these strain combinations. The major difference is in the hematocrit profiles; DBA/2J plus (DBA/2J x C3H.A)F1 parabionts do not show the anemia-polycythemia characteristic of DBA/2J plus (DBA/2J x C3H/HeJ)F1 pairs (Figures 5 and 10). Clearly though, in most ways the stimulus of the K through I-E subregions of H-2K is almost as strong as the stimulus of the entire H-2K haplotype from K end to D end.

Just as clearly, the D end alone, presented to DBA/2J partners by (DBA/2J x C3H.OH)F1 partners in parabiosis, is very weak. Most parabionts survive, a complete takeover never occurs, and there is no hematocrit divergence. Assays of cell mediated immunity were not done, since the immunostimulatory ability of H-2D is very weak (Nisbet and Edwards, 1973; Klein, 1972).

While H-2 determines the most important "target" antigens, a minor role at best may still exist for non-H-2 antigens. Substituting other H-2K strains for C3H/HeJ in parabiosis did not significantly affect survival or takeover (Figure 11), incidentally eliminating the Mls locus from a role in this system, when DBA/2J is the responder. Furthermore, the mortality of DBA/2J plus (DBA/2J x C3H/HeJ)F1 pairs, about 56%, equals the sum of the mortalities of the DBA/2J plus (DBA/2J x C3H.A)F1 pairs (about 42%) and the DBA/2J plus (DBA/2J x C3H.OH)F1 pairs (aboput 17%). This may indicate that these components of the

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H-2 complex are responsible for all the mortality of parabiosis, but further analysis is impossible without other C3H congenics. If the non-H-2 genetic background of the C3H or CBA mice used here contributes to any of the mortality, the contribution is very small. Also, non-H-2 genes that C3H and the two CBA lines share may be important but not apparent.

BALB/c mice respond far more strongly to H-2K-bearing F1 partners than do DBA/2J mice (Figure 11). Whatever the reason for this, it is apparently dominant over the DBA/2J phenotype since (DBA/2J x BALB/c)F1 partners respond almost as strongly to (DBA/2J x H-2K)F1 partners as BALB/c mice.

The overall conclusion from this work on genetic influences in parabiosis is that there appears a reasonably strong relationship between the degree of histoincompatibility and both parabiont survival and the extent of the takeover process. The H-2 complex is of central importance and within H-2, the subregions from K to I-E appear more immunogenic than the H-2D subregion, which is entirely consistent with earlier work both with parabiosis (Nisbet and Edwards, 1973) and other studies (McKenzie and Snell, 1973; Klein, 1972; Rychlitova, et. al., 1970). This effect is particularly prominent with DBA/2J responding parental partners. When the various C3H strains were tried as parental partners (C3H/HeJ, C3H.SW, C3H.NB) parabiosed to DBA/2J-derived F1 hybrids, or when BALB/c was

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the parental partner, none of the parabionts survived (Drell and Wegmann, unpublished data, and Figure 11); therefore, non-H-2 genes influence survival as well.

What this may resemble is the situation encountered in spontaneous enhancement models (Section IV.4). Variations both with histocompatibility and with strain occur both in parabiosis, as just discussed, and in enhancement as well (Sections V.2, V.3). Thus as a mechanism involved in parabiont survival, enhancement merits consideration.

The potential for reactivity to F1 alloantigens persists in parabiosed mice despite the in situ tolerance. Active effector cells are not detectable within the limits of sensitivity of the assays employed (Tables 5 and 7) and our tests for suppressor activity mediated by spleen cells are negative. Anti-F1 antibody is present and this is the greatest similarity with the situation in enhancement. This antibody, upon analysis with congenic C3H target cells in vitro (Figure 21), is directed at antigenic determinants of the K to I-E subregions of the H-2k haplotype, the same set of determinants which are crucial for the takeover reaction. This antibody seems largely, although perhaps not exclusively, to be IgM, which can enhance for tumor grafts (Fuller and Winn, 1973; van Breda Vriesman, et. al., 1975; Bubenik, et. al., 1965).

Several studies, reviewed in Section IV.4., have noted the presence of anti-donor cell mediated immunity in

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"spontaneously enhanced" animals (Bildsoe, et. al., 1971; Paris, et. al., 1978) and two reports note the presence in a kidney grafting model of all the reactivities here described for parabiosed mice, including antibody (Russell, et. al., 1975; 1978a). In addition, these models all appear to involve "progressive" enhancement since skin is ultimately accepted by these grafted recipients. The only reports of enhancement benefitting normal tissue other than skin, heart, kidney, or endocrine grafts are those of Poole, et al., (1976) who enhanced entire rat hind leg allografts, and Gutmann, et. al., (1978) who enhanced allografts of rat muscle tissue. It may be only an extension in degree from enhancement of these types of graft to enhancement of the graft of an entire mouse. This may be more reasonable when viewed in the light of the takeover which removes the potent stimulatory cells of the lymphoid and erythropoietic series.

The actual function of the antibody in parabiosed mice in unclear. Since, in the tests described here (Figures 19-21, and Table 8), the target spleen cells and RI tumor cells (Olivotto and Bomford, 1974) are both of lymphoid derivation, antibody activity against non-lymphoid, tissue, or organ specific antigens would not be detected. Also, <u>in vitro</u>, IgM antibodies are much more easily detected by complement mediated cytotoxicity than are IgG antibodies (Winn, 1962) so these <u>in vitro</u> tests may not reveal the true amounts or relative proportions of the classes present <u>in vivo</u>.

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One function the antibody may have is to effect the takeover reaction but in this connection, it must be emphasized that all our studies to date have been performed on long-term surviving parabionts. The takeover mechanism is not known, other than that parental immunocompetence is required (Figure 3). Thus, we do not know whether the antibody plays an early role in the takeover or is responsible for survival. In all of 12 pairs analyzed to date, however, this antibody was found and in titers of 1:10 to 1:20 so it appears characteristic. If immunological enhancement via antibody is the mechanism maintaining unresponsiveness in situ, it is unlikely this is due to efferent action since active effector cells are not present. Thus an afferent or central mode of action may be more likely, perhaps considerably aided by the takeover, which would inhibit recognition of F1 alloantigens by the otherwise responsive parental cell mediated immune system.

The specificity of the parabiont antibody must be significant. Our data indicate that the antibody is directed largely against determinants coded for by one or more genes in the K through I-E subregions of the H-2k haplotype (Figure 21); reactivity against H-2D end loci (shared by C3H.OH with C3H/HeJ) is not a detectable component of the parabiont antibody activity, nor is there antibody activity against non-H-2 genetic determinants represented on C3H.NB target cells. Here, limitations are imposed by the available "library" of congenic C3H mice, so the analysis of antibody

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specificity is incomplete and the correlation with the other consequences of these specific alloantigenic disparities is necessarily rough. Furthermore, re-analysis must be done on the three other high-surviving congenic strain combinations of parabionts, those using (DBA/2J x C3H.NB)F1, (DBA/2J x C3H.A)F1, and (DBA/2J x C3H.OH)F1 mice.

If this antibody is the agent of the takeover reaction, then very possibly the DBA/2J plus (DBA/2J x C3H.NB)F1 and (DBA/2J x C3H.A)F1 strain combinations will possess it, while the DBA/2J plus (DBA/2J x C3H.OH)F1 parabionts will not. The correlation between the specificity of the antibody seen in the DBA/2J plus (DBA/2J x C3H/HeJ)F1 parabionts, and the presence of the takeover reaction in all those parabiont combinations in which the F1 partner presents incompatibilities in the K to I-E interval of the H-2 complex, suggests that this is the role the antibody may play. In the DBA/2J plus (DBA/2J x C3H.OH)F1 parabionts, there is no incompatibility at H-2 except the relatively weak H-2D end and this might not be strong enough to stimulate sufficient or effective antibody. It may be the case in these parabionts that the histoincompatibility is so weak that an entirely different non-antibody-mediated mechanism occurs.

The normal rejection time of F1 parabiont skin grafts on normal DBA/2J recipients is consistent with the observed rejection of enhanced tissues upon retransplantation to

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unenhanced hosts (Jenkins and Woodruff, 1973; Fabre and Morris, 1972b; Stuart, et. al., 1969). Rubin (1959) first observed that C3H/HeJ skin grafts onto DBA/2J partners of DBA/2J plus (DBA/2J x C3H/HeJ)F1 parabionts were not rejected and he suggested immunological enhancement as a mechanism. Others studying spontaneous enhancement have found that donor strain skin grafts can survive much longer (Skoskiewicz, et. al., 1973) or even permanently (Salaman, 1971a) on previously grafted recipients. Although the cumulative literature on tissue graft enhancement does not make clear why such variability of skin grafts is seen, in the parabiont model it is clear that the immunogenicity of F1 skin is intact (Table 9).

Although considerable work remains to be done on the cell injection takeover model, it is clear that a large number of parental spleen cells, capable of reacting against the recipient F1's alloantigens, can cause the takeover by themselves. It is tempting to equate the takeover caused by cell injection with the takeover following parent-F1 hybrid parabiosis. However, caution is advisable until the cell injection chimeric mice are characterized for immune status. All of the cell injection chimeras produced to date and in which a complete takeover has occurred have been parent into F1 chimeras, incorporating total haplotype differences. A more thorough genetic analysis remains to be done. The C3H.A and C3H.OH strains could be used to ask whether takeover of (DBA/2J x C3H.A)F1 and (DBA/2J x C3H.OH)F1 hybrids by DBA/2J

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spleen cells will occur. If the hypothetical analogy with parabiosed mice holds, cell injection may not result in takeover of the latter F1 hybrid recipients. An equally important approach would address whether the injected cells would cause takeover if the cells were unable to respond to H-2 antigens of the recipients; spleen cells from established bone marrow chimera mice would be suitable for this. A further refinement would be to construct different types of bone marrow or fetal liver chimeras and, using them as donors of spleen cells, with the different available F1 hybrids, ask (to a limited extent) whether the left hand end or the right hand end of the H-2 complex provides sufficient (or necessary) stimulus for this form of takeover, as the left hand end of H-2 seems to do for parabiosis-mediated takeover.

The use of the hybridoma to "ease" the takeover by allowing a fivefold decrease in the number of spleen cells needed to accomplish it, constitutes an easy, simple, and sure way of generating chimeric mice. Again, until the immune status of these mice is checked, analogies with parabiont F1 mice should be made guardedly. In these pretreated and spleen cell injected mice, the hybridoma used was directed solely at the H-2K(k) determinant. This antigen is present throughout the tissues of the F1; what effects, if any, this may have on cells other than erythropoietic precursors (the only ones <u>known</u> to have been functionally eliminated) remains to be determined.

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In none of these cell injection chimeras, whether pretreated with hybridoma or not, was there any overt sign of early graft-versus-host disease. Survival of these chimeras was not perfect, however, as can be seen in Table 10; given large numbers, reliable survival figures, which can be compared to the known survival figures for parabiosed mice of the same combinations, can be accumulated. Also, protocols can be worked out which might reduce the mortality to neglegible quantities. If so, the innocuous takeover which results might serve as a very easy and hopefully useful model for the study of bone marrow cell grafting.

Thus parabiosis is an experimental model in which unresponsiveness across H-2 barriers is maintained despite the retention of the potential for alloreactivity towards the H-2 antigens of the "tolerated" partner. The immunogenetic relationship of responding partner to "tolerated" partner is crucial, with H-2 determinants in the K to I-E interval serving as critically important stimuli both for survival and takeover of the lymphoid and erythropoietic systems. The takeover reaction, perhaps aided by the presence of antibody activity to these same "tolerated" alloantigens, enforces the absence from the parabionts of F1 antigens in their most immunostimulatory and therefore potentially deadly form, on circulating lymphoid and erythropoietic cells. If the antibody plays a role in maintaining this non-reactivity <u>in situ</u> by reducing the immunogenicity of the F1 partner, this may point the way

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towards a better understanding of what comprises immunogenicity for all tissues, as well as providing possible strategies for deliberately reducing it for tissue grafts.

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